Attorney Docket # 4493-19C/RCE

Patent

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#16

In re Application of

Shalom Z. Hirschman et al.

Serial No.: 09/316,624

Filed: May 21, 1999

For: A Method For Treating Autoimmune Diseases

Examiner:
Group Art:

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Yunling Ren
Name of applicant, assignee or Registered Representative

Signature

April 28, 2003
Date of Signature

Board of Patent Appeals and Interferences
Washington, D.C. 20231

REPLY BRIEF

SIR:

This is responsive to the Examiner's answer dated February 26, 2003 pursuant to 37 CFR § 1.193 (b)(1).

Applicant repeats and emphasizes that the present invention is directed to a method of treating rheumatoid arthritis by administering a therapeutic composition, namely Product R., which is made and defined by a specific method described in the present application. There has been no prior art that teaches how to administer Product R to treat a patient suffering from rheumatoid arthritis. The Examiner maintains her rejection to the present invention as being anticipated by the U.S. Pat. No. 5,849,196 (the '196 patent) to Kochel is based on her misunderstanding of the facts and the issues relevant to the determination of anticipation.

I. <u>The Examiner Failed to State A Basis for Her Rejection to the Claim Language "an effective symptom ameliorating amount of Product R in a range from about 2.5 microliters to about 40 microliters per kilogram of body weight per day"</u>.

In asserting that the claim language "an effective symptom ameliorating amount of Product R in a range from about 2.5 microliters to about 40 microliters per kilogram of body weight per day" under 35 U.S.C. 112, second paragraph, the Examiner has failed to establish a basis underlining her arguments.

The Examiner argues that "specification provides two methods for making Product R but it is unclear that the two resulting products have the same level of activity". However, applicant, in a response to a Final Action dated May 17, 2001, expressly stated that the term "Product R" is now limited to Method I, on page 10 of the present specification, which reads:

> **Method I For Preparing Product R**
>
> Suspend about 35.0 g of casein, about 17.1 g of beef peptone, about 22.0 g of nucleic acid (RNA), about 3.25 g bovine serum albumin in about 2.5 liters of water for injection USP at about 3 to 7 °C in a suitable container and gently stir until all the ingredients have been properly wet. Carefully add while stirring about 16.5 g of sodium hydroxide (reagent grade ACS) and continue stirring until sodium hydroxide completely dissolved. Autoclave at about 9 lbs pressure and 200 - 230 °F for a period of time until RNA is completely digested, for example, about 4 hours. At the end of the period, the autoclave is stopped and the reaction flask and contents are permitted to slowly cool to ambient temperature. Then cool for at least six hours at about 3-8 °C. The resulting solution is filtered through 2 micron and 0.45 micron filters using inert gas such as nitrogen or argon at low pressure (1-6 psi). In a similar manner the solution is filtered again through 0.2 micron pyrogen retention filters. The resulting filtrate is sampled and assayed for total nitrogen. A calculation is then performed to determine the quantity of cooled water for injection to be added to the filtrate to yield a diluted filtrate with a nitrogen content between about 165-210 mg/ml, the final volume is approximately 5 liters. The pH is then adjusted with either concentrated HCl (reagent grade ACS) or 1.0 normal NaOH to about 7.3 - 7.6 range. The diluted solution is then filtered again through 0.2 micron filters with inert gas at low pressure. The final filtrate is then filled and sealed into 2 ml glass ampules while in an inert gas atmosphere. The ampules are collected and autoclave for final sterilization at 240 °F and 20 to 30 pounds pressure for about 30

minutes. Following the sterilization cycle, the ampules with Product R are cooled and washed.

All quantities are subject to plus or minus 2.5% variation for pH, volume, and analytical adjustments.

Thus, the Examiner's objection to the recitation of two methods for making Product R has become moot long ago.

The Examiner further argues that applicant has not establish any type of activity of Product R nor provide evidence of activity of Product R, but ignores that the claimed invention of the present application is a method of treating auto immune disease Product R, and that the specification has provided ample evidence supporting the claimed subject matter. In the instant case, the preparation of Product R is described in great detail on page 10, as shown above; the dosages and method for treating rheumatoid arthritis by parenterally administering Product R is described from pages 12 to 20; and the biological activity of Product R and the results of the treatment of rheumatoid arthritis are presented in Table 1 on page 19 and Figs. 1A, 1B, 1C, and 2-5. A person of ordinary skill in the art, following these teaching, will be able to make Product R and administer Product R to a patient.

The Examiner appears to have not referred to the information provided in the specification and the applicant's remarks that direct Examiner's attention to the place in the specification where such information is located. By repeatedly requiring applicant to provide the same information that has already been provided, the Examiner has failed to state a basis for the rejection.

The Examiner also argues that the wide range of volumes to be administered would not allow a person of ordinary skill in the art to estimate proper dosages of Product R,

based on her erroneous presentation of the fact that Product R is given to the patient from 2.5 μl (microliter) to 1 ml (milliliter). The truth is that the amount of Product R to be administered to a patient is described by either <u>microliters/Kg/day (e.g. 2.5 microliters per kilogram of body weight per day, see claim 1</u>) or <u>milliliters/patients (e.g. 1 milliliter per patient</u>). Nowhere in the present application does applicant ever describe the dosages of Product R ranging from 2.5 μl (microliter) to 1 ml (milliliter). Thus, based on applicant's correct and accurate dosages for Product R, namely 2.5 μl /Kg/day which equals about 0.175 milliliters per person or about 1/5 of 1 milliliter per patient, assuming such person weights about 70 kilograms (about 126 pounds), the claimed range is <u>from about 1/5 milliliters to about 1 milliliters</u>, not from 2.5 μl (microliter) to 1 ml (milliliter). Thus, applicant's range is not unduly wide by any standard.

Again, the Examiner has failed to establish a basis for her dosage rejection due to her misrepresentation of the fact.

II. <u>Examiner Has Not Establish that Each and Every Requirement of the Present Invention is Met by the '196 Patent.</u>

To anticipate the present invention, the '196 patent must teach <u>each and every element</u> of the presently claimed invention. Even assuming that the Examiner's baseless assumption that Product R and Kochel's composition are same is correct, the '196 patent does not teach a method of administering any product, including Kochel's composition, to treat rheumatoid arthritis.

The Examiner asserts Kochel teaches how to administer his composition to treat rheumatoid arthritis by taking the facts disclosed in the '196 patent out of context and making up somewhat new facts that have never existed.

The Examiner has acknowledged that Kochel teaches how to divided the conventional composition into a high molecular weight fraction and a low molecular weight fraction. In columns 13 and 14 of the '196 patent, Kochel describes the administration route of his high molecular weight fraction for treating certain diseases, namely viral infections, but NOT rheumatoid arthritis. Nowhere in the '196 patent does Kochel ever discuss how to use his low molecular weight fraction to treat any diseases. The only place where Kochel mentions that the low molecular weight fraction may be useful for treating auto immune diseases including rheumatoid arthritis is in column 3, line 1-11, which reads as follows:

> "Further, although the lower weight active components (MW <8-15 kDa) of the composition are not effective as antiviral agents, they are effective in treating auto immune diseases such as non-Hodgkins Lymphoma, Adult Onset Leukemia, AIDS, Lupus, Scleroderma, Epstein Barr virus, Cytomegalovirus, Chronic Fatigue Syndrome, Candidiasis, Rheumatoid and Osteo Arthritis, etc. Thus, the active components of the conventional composition may be segregated according to molecular weight and the different resulting groups of components may be selectively used to treat different viruses and auto immune diseases accordingly." (emphasis added)

Such teaching can hardly enable any person of ordinary skill in the art to use a drug to treat a rheumatoid arthritis patient. The above passage is, pure and simple, an invitation to experimentation. It is hardly an anticipation of anything.

By arguing that Kochel teaches how to treat rheumatoid arthritis because he discloses how to treat viral infections with his high molecular weight composition that has no activity in the treatment of rheumatoid arthritis, the Examiner in an effort to create an anticipation has misplaced the facts to where such facts do not belong.

Thus, just based on this alone, the '196 patent cannot anticipate the present claimed invention which, at a minimum, requires an administration route and the dosages.

Further, Kochel's composition in the '196 patent is NOT product R, which applicant has provided ample evidence supporting applicant's position in the initial appeal brief.

Applicant's position is further supported by the fact that both Product R and the Kochel's composition have been awarded with separate patents by the Patent Office, respectively, based on the distinct natures and properties of these two products. See U.S. Pat. Nos. 5,849,196 and 6,303,153 (the '153 patent) attached here as Appendix A and Appendix B. Therefore, these two products are patentably distinguishable. It would be entirely inconsistent for the Patent Office to first acknowledge that the two products are different by granting unrelated separate patents to each of them, and now to take an opposite position by asserting the two products are patentably indistinguishable.

A simple comparison between the description of Product R in the present application (on page 10) and the description of Product R in the '153 patent (column 10, line 51 to column 11, line 17) shows that the descriptions are verbatim the same. Therefore the Product R in the present invention is identical to the Product R in the '153 patent.

The Examiner speculates that Product R might be the same as Kochel's composition disclosed in the U.S. Pat. No. 6,303,153 (the '153 patent) by stating that only quantities but no concentrations of the starting materials of the instant application and the starting materials disclosed by Kochel are provided so as to be able to compare these two products. However, the Examiner ignores the fact that both concentrations of the starting materials in the instant application and the starting materials disclosed by Kochel are indeed expressly provided. See page 10 of the present application and column 5, lines 9-20 of the '196 patent.

In the instant application, the concentration of the starting materials is described in the method for preparing Product R, as follows:

> **Method I For Preparing Product R**
>
> Suspend about 35.0 g of casein, about 17.1 g of beef peptone, about 22.0 g of nucleic acid (RNA), about 3.25 g bovine serum albumin in about 2.5 liters of water for injection USP at about 3 to 7 °C in a suitable container and gently stir until all the ingredients have been properly wet. Carefully add while stirring about 16.5 g of sodium hydroxide (reagent grade ACS) and continue stirring until sodium hydroxide completely dissolved... . the final volume is approximately 5 liters... . (Emphasis added).

Spec. p 10.

In the '196 patent, the concentration of Kochel's starting materials are described as follows:

> First, the indicated quantities of the following components are mixed into ten liters of distilled water under slow stirring:

RAW MATERIALS	AMOUNTS	WEIGHT PERCENTAGE
Casein	250 grams	43.9%
Blood albumin	15 grams	2.6%
Beef peptone	150 grams	26.3%
Nucleic acid (RNA)	80 grams	14.0%
Sodium hydroxide	75 grams	13.2%

U.S. Pat. No. 5,849,196, Col. 5, ll 9-20.

Applicant has also made a table to compare the concentrations of the starting materials in both products based on the above facts in applicant's Appeal Brief submitted previously, which shows that the concentrations of the starting materials in the two products are materially different. See the Appeal Brief, page 4.

Since the above facts clearly shows that the concentrations of starting materials of Product R and the Kochel's composition are materially different, the Examiner's assumption

that the two products <u>might be the same</u> because the two concentrations "might be" the same must fail.

That Product R and Kochel's composition are different products has also been confirmed by the analysis of the physical, chemical and biological properties of the two products. The results of the analysis were shown in applicant's appeal brief, page 5.
Again, the properties of Product R are inherent, even if the application does not expressly mention the features. *Tyler Refrigeration v. Kysor Industrial Corp.*, 777 F.2d, at 689.

The Examiner argues that no particular properties are clearly associated with the term "Product R", ignoring that applicant has defined Product R by its process. In other words, Product R is process sensitive. If one alters that particular process, one alters the Product. That is an important property of Product R. The language defining Product R can be found on page 10, line 2 of the instant application. Therefore, the term "Product R" is not subject to any other interpretation.

The Examiner's discussion on the scope and meaning of a product by process claim is irrelevant to any issue here. There is no product claim in the instant application. All claims are directed to a method of using Product R.

For the above reasons and reasons applicant stated in the initial Appeal Brief, the Board should allow all pending claims in the instant application.

It is believed that no fees or charges are required at this time in connection with the present application; however, if any fees or charges are required at this time, they may be charged to our Patent and Trademark Office Deposit Account No. 03-2412.

Respectfully submitted,

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US005849196A

United States Patent [19]

Kochel

[11] **Patent Number: 5,849,196**

[45] **Date of Patent: Dec. 15, 1998**

[54] **COMPOSITION CONTAINING PEPTIDES AND NUCLEIC ACIDS AND METHODS OF MAKING SAME**

[75] Inventor: **Bonawentura Kochel**, Wroclaw, Poland

[73] Assignee: **Immune Modulation Maximum**, New York, N.Y.

[21] Appl. No.: **726,650**

[22] Filed: **Oct. 7, 1996**

[51] Int. Cl.[6] **B01D 61/24**; A61K 35/14; A61K 35/20; A61K 35/72

[52] U.S. Cl. **210/651**; 424/520; 424/529; 424/535; 514/2; 514/7; 514/44

[58] Field of Search 435/91.1; 514/2, 514/7, 44; 530/300, 350, 360, 363; 536/23.1; 210/651; 424/520, 529, 535

[56] **References Cited**

U.S. PATENT DOCUMENTS

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Hanvey et al. "Antisense and Antigene Properties of Petide Nucleic Acids" Science 258: 1481–1485, Nov. 1992.

Nielsen et al. "Peptide Nucleic Acid (PNA). A DNA Mimic wiht a Peptide Backbone" Bioconjugate Chem. 5: 3–7, Feb. 1994.

Nielsen et al. "Peptide Nucleic Acids (PNAs): Potential Antisense and Anti–Gene Agents." Anti–Cancer Drug Design 8: 53–63, Aug. 1993.

"Peptide Nucleic Acids Stimulate Gamma Interferon and Inhibit the Replication of the Human Immunodeficiency Virus", by Shalom Z. Hirschman and Chey Wei Chen, in Journal of Investigative Medicine, vol. 44, No. 6, Aug. 1996, pp. 347–351.

Compilation manual for Clinical Symposium On Viral Diseases Demonstrating The Anti–Viral Biotic Properties Of The Drug Reticulose, 01 Sep. 1960, Miami, Florida.

"For The Viral Infection", informational compilation by Advanced Viral Research Corp., publication date not included.

Primary Examiner—George C. Elliott
Assistant Examiner—Thomas G. Larson
Attorney, Agent, or Firm—Carrier, Blackman & Associates, P.C.; Joseph P. Carrier; William F. Esser

[57] **ABSTRACT**

An improved composition containing peptides and nucleic acids has active components, i.e., which heighten the phagocytic activity f neutrophils, consisting of molecules with a molecular weight of at least 8 kDa, and preferably at least 15 kDa. The active components comprise peptides without aromatic portions and will absorb light at an absorption band of $\Delta\lambda$=200–235 mn, λ_{max}=205 nm, in the UV spectrum. The composition is nontoxic and is formulated using casein, blood albumin, beef peptone, nucleic acid (RNA) and a base such as sodium hydroxide. The composition stimulates phagocytic activity of neutrophils if used at sufficient concentrations. A separate composition is obtained using the same components of manufacture, but filtering or centrifuging the composition to a molecular weight of <8–15 kDa which inhibits phagocytic activity of neutrophils for application in treating auto immune diseases.

16 Claims, 3 Drawing Sheets

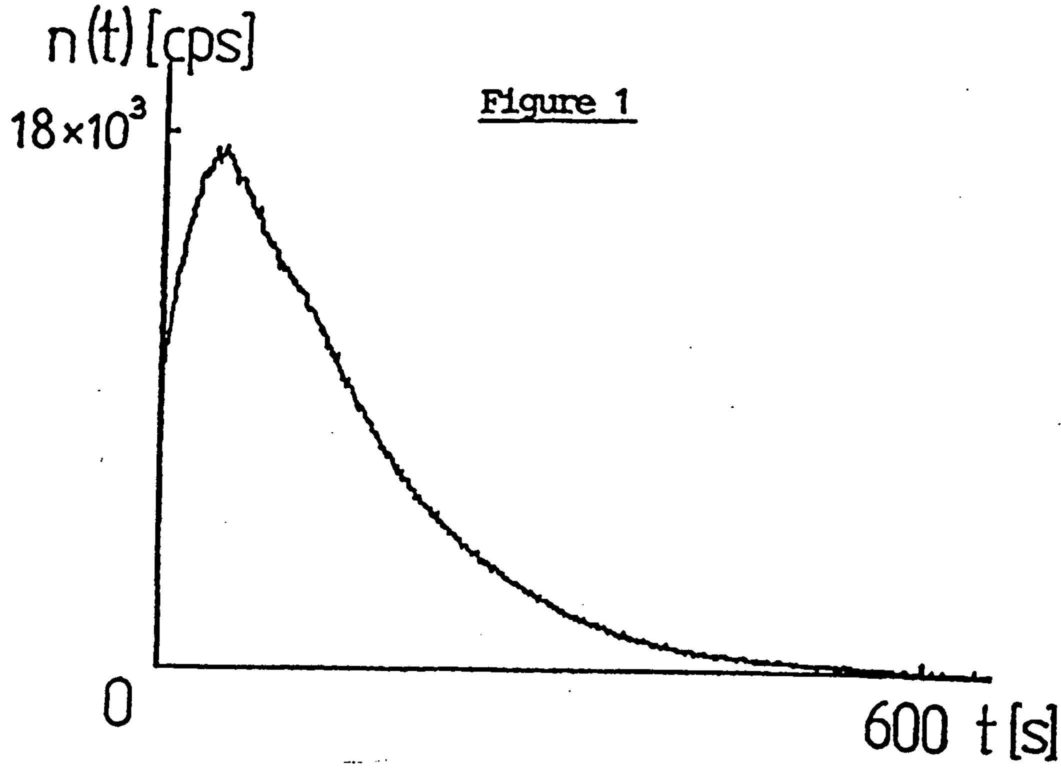

Figure 1

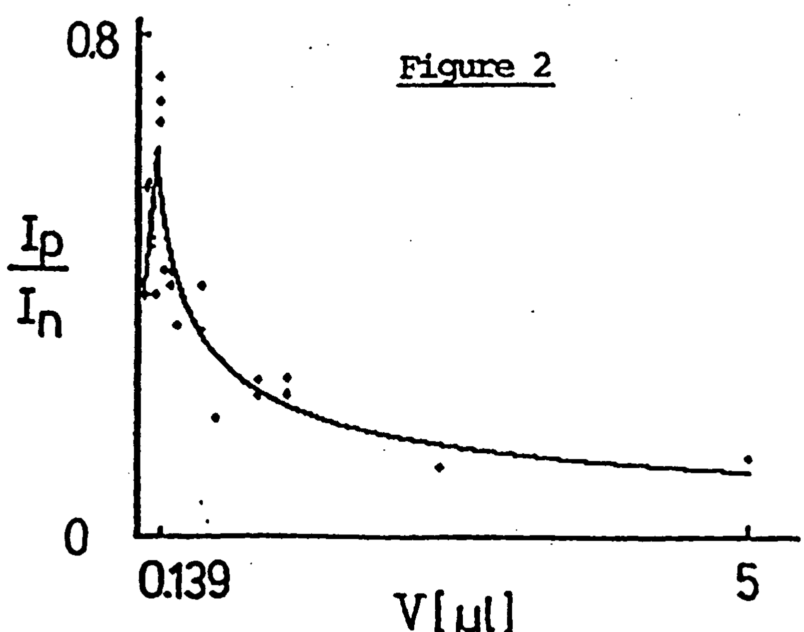

Figure 2

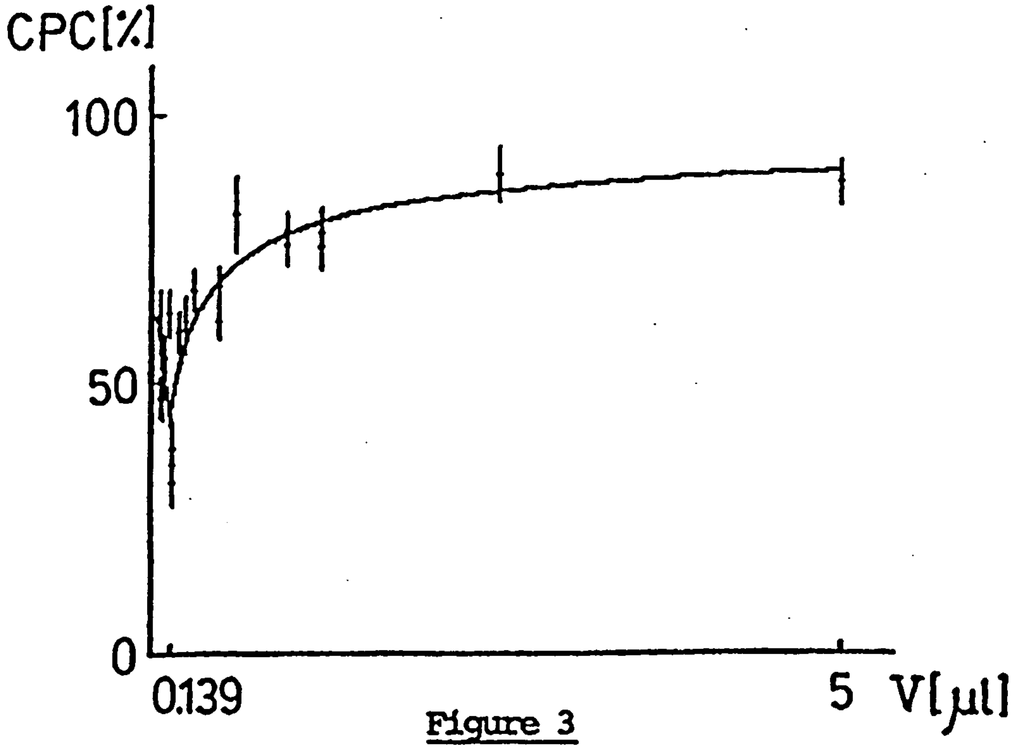

Figure 3

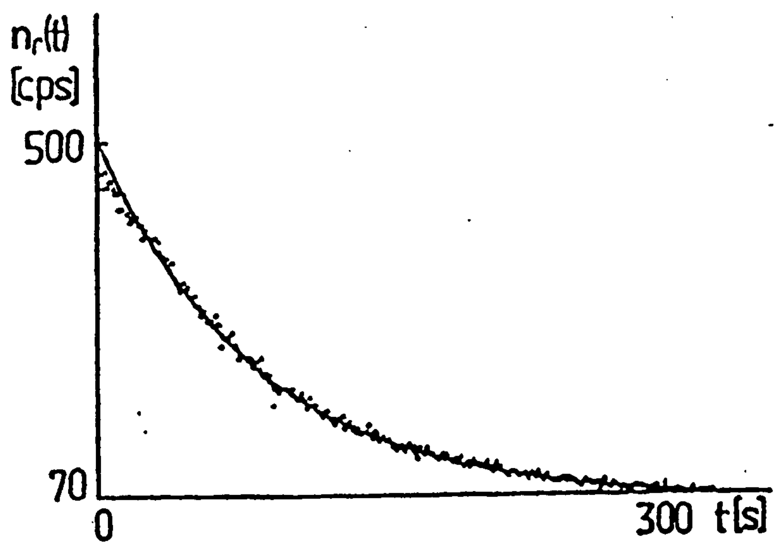

Figure 4

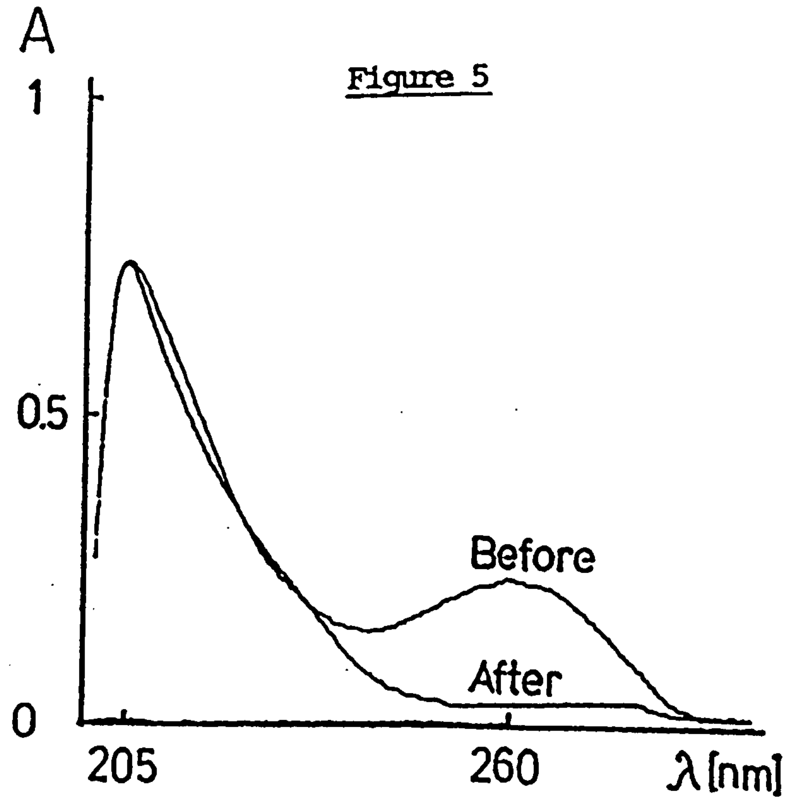

Figure 5

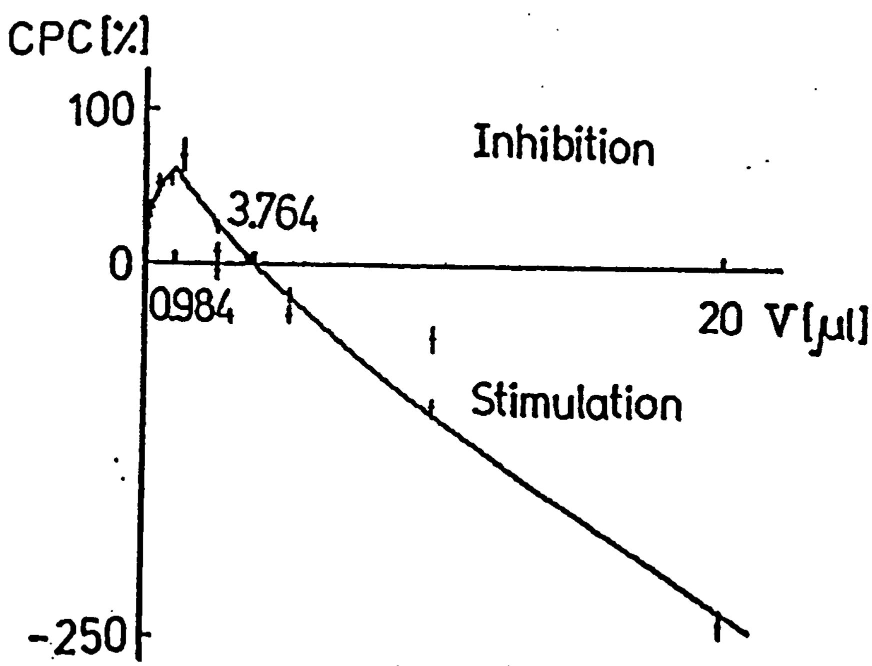

Figure 6

COMPOSITION CONTAINING PEPTIDES AND NUCLEIC ACIDS AND METHODS OF MAKING SAME

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to a novel composition containing peptides and nucleic acid which is useful as antiviral agent and as an agent useful in treating auto immune diseases, and to methods of formulating and utilizing same. More particularly, the invention pertains to such a composition which is modified to have an improved ability to stimulate phagocytosis in humans for treatment of viruses and the like, and to methods of formulating and utilizing same.

2. Description of Relevant Art

In the art there is at least one conventionally known composition containing peptides and nucleic acids distributed under the trademark Reticulose™, which has been used as antiviral agent for humans in relation to treatment of viral infections/diseases such as influenza, herpes, infectious mononucleosis, hepatitis A and B, and most recently HIV. The known composition is referred to as "conventional composition" hereinafter. See Anderson R. H. & Thompson R. N., *Treatment of Viral Syndromes With A Lipo-protein Nucleic Acid Formulation (Reticulose)*, VIRGINIA MED. MONTH. 84, 347–353, 1957; Wegryn S. P., Marks R. A. and Baugh J. R., *Herpes Gestations*, Am J. Obst. & Gynecol. 79.812–814, 1960; Reynolds M. R., *Generalized Vaccinia Successfully Treated With Lipoprotein-Nucleic Acid Complex (Reticulose)* Arch Pediatrics 77.421–422.1960; Medoff L. R. *Use Of A Lipoprotein-Nucleic Acid Formulation In Treatment Of Infectious Mononucleosis*, Clin. Med. 69, 1–4, 1962; Catterall R. A. *A New Treatment Of Herpes Zoster, Vaccinia And Chicken Pox*, J. Roy. Coll. Gen. Practit. 19.182–183, 1970; Friedland B., *In vitro Antiviral Activity Of A Peptide-Nucleic Acid Solution Against The Human Immonodeficiency Virus And Influenza A Virus*, J. Royal Soc. Health 111, 170–171, 1991; Hirschman S. Z. and Chen W., *Peptide Nucleic Acids Stimulate Gamma Interferon And Inhibit Replication Of Human Immunodeficiency Virus*, Proc. Biomedicine '96., Washington D.C., U.S.A., May 3–6, 1996. Thompson R. M., *A Lipo-Protein Nucleic Acid Complex In The Treatment Of Radiation Injury*, The Military Surgeon, 110, 51–58, 1952; Strickland W. N., *Summary Of Peptide-Nucleic Acid Studies Conducted At The University of Wisconsin Biotechnology Center*, Reticulose, Commonwealth Pharmaceuticals, Trenton, 1995, pp. 19–35; Friedland, B., *In Vitro Antiviral Activity of a Pepti-Nucleic Acid Solution Against The Human Immunodeficiency Virus and Influenza Virus*, J. ROY SOC. HEALTH, V. 111, No. 5, PP170, 171, 1991; and Cohen M. *The Efficacy of a Pepti-Nucleic Acid Solution (Reticulose™) For The Treatment of Hepatitis A and Hepatitis B-A Preliminary Controlled Human Clinical Trial*, J. ROY SOC. HEALTH, V. 112, No. 6, PP. 266–270 1992.

The conventional composition, also generally referred to as nucleophosphoprotein and a lipoprotein nucleic acid solution, was originally conceived by Dr. Vincent LaPenta around 1934 and was commercially available in the U.S. for a period ending in 1962. The conventional composition is known to be formulated through a mixture of casein, beef peptone, ribonucleic acid (RNA), beef serum (blood) albumin, sodium hydroxide and distilled water which is processed through heat, pressurization and filtration to a solution that is of such a small molecular weight as to be compatible with any human blood type, as discussed further hereinbelow. Essentially, the conventional composition is a complex solution of peptides and nucleic acids in which nucleic acid fragments are associated or possibly associated with short chain peptides, and wherein the molecular weight of the active components ranges from approximately 1 to 25 kDa. Presently, the conventional composition is still manufactured according to its original formulation by Advanced Viral Research Corp., in Miami, Fla.

Although the exact nature of the antiviral activity caused by complexes of peptides and nucleic acids such as the conventional composition is unknown, it appears to act either by an ability to inhibit the viruses or by alteration of a host cell response in preventing virus multiplication, and a capacity to increase antiviral, antibody response in humans, which exerts a positive therapeutic effect in both acute and chronic infection. Also very significantly, the conventional composition has been shown to be substantially free from side effects and systemic toxicity, unlike most other antiviral agents, including AZT and beta interferon.

Although the conventional composition has certain advantageous characteristics as discussed above, its effectiveness as antiviral agent is known to be limited and erratic, especially when compared to other known antiviral agents including AZT, Ribavarin, Dideoxyadenosine (DDI) and Dideoxycytidine (DDC). It thus remains a desideratum in the art for an antiviral agent which is, like the conventional composition, substantially free of ill side effects and systemic toxicity, but which also has improved effectiveness as an antiviral agent in comparison to the conventional composition.

SUMMARY OF THE INVENTION

The present invention has been developed to fulfill the above-discussed desiratum in the art.

According to the invention there is provided a composition containing peptides and nucleic acids whose active components consist essentially of molecules having a molecular weight of at least 8 kDa. Preferably, the formulation comprises peptides without aromatic portions, and has an absorption band in the interval of $\Delta\lambda$=200–235 nm, with a maximum absorbtion at λ_{max}=205 nm. Most preferably, the formulation according to the invention will stimulate a phagocytic activity of neutrophils above a predetermined quantity of the formulation.

Applicant has determined that the conventional composition, which contains active components with a molecular weight ranging from approximately 1 to 25 kDa, exhibits a phenomenon of inhibition of neutrophil phagocytic activity in humans by different groups of active components contained therein, i.e., applicant has discovered that a concentration dependent inhibition is caused by small nucleic acid fragments associated with peptides containing aromatic amino acids (MW<8–15 kDa), working as phagocytosis inhibitors. Conversely, the stimulation is caused by heavier molecules (MW>8–15 kDa), including peptides without aromatic components, which stimulate phagocytosis. Based on such discovery, applicant has modified the conventional composition by removing therefrom those active components with the molecular weight <8–15 kDa so that the resulting or modified composition exhibits mainly stimulatory effects on the phagocytic activity of neutrophils. Specifically, applicant has discovered that the composition according to the invention function as priming factors which convert neutrophils to a status more "respondent" to external stimuli such as N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP).

Further, although the lower weight active components (MW<8–15 kDa) of the composition are not effective as antiviral agents, they are effective in treating auto immune diseases such as non-Hodgkins Lymphoma, adult onset Leukemia, AIDS, Lupus, Scleraderma, Epstein Barr Virus, Cytomegalovirus, Chronic Fatigue Syndrome, Candidiasis, Rheumatoid and Osteo Arthritis, etc. Thus, the active components of the conventional composition may be segregrated according to molecular weight and the different resulting groups of components may be selectively used to treat different viruses and auto immune diseases accordingly.

According to another important aspect of the invention there is also provided a method of preparing a composition containing peptides and nucleic acids, comprising the steps of:

combining casein, blood albumin, beef peptone, nucleic acid and a base such as sodium hydroxide in a solution of distilled water; processing the solution under elevated temperature and elevated pressure to associate nucleic acid and peptide components of the solution; and filtering or centrifuging the processed solution to remove active components having a molecular weight of less than 8 kDa. Preferably, the filtering step will be performed in multiple stages, including an initial filtering or centrifuging stage comparable to that used in making the conventional composition, which results in a solution having active components of varying molecular weights ranging from approximately 1 to 25 kDa, and a secondary filtering or centrifuging stage in which the thus filtered solution is further filtered through a semipermeable membrane such that the resulting formulation contains active components having molecular weight exclusively in a higher range of approximately 15–25 kDa.

Again, it is preferred that the active components of the composition thus processed will include peptides having no aromatic components, and the active ingredients will absorb light in a band of $\Delta\lambda$=200–235 nm, λ_{max}=205 nm, A=0.06 in the UV spectrum.

Still further, according to another aspect of the invention there is provided a method of utilizing a composition containing peptides and nucleic acids whose active components consist essentially of molecules having a molecular weight of at least 8, and most preferably 15, kDa as antiviral agent, comprising the steps of:

(a) treating a virus infected patient with a first amount of an aqueous solution of the composition per day over a first period of days;

(b) sequentially thereafter passing a second period of days without additional patient treatment of the composition;

(c) sequentially thereafter repeating steps (a) and (b) in a cyclic pattern;

(d) sequentially thereafter treating the patient with a second amount of an aqueous solution of the composition per day over a third period of days;

(e) sequentially thereafter passing a fourth period of days without additional patient treatment of the composition; and

(f) thereafter testing the patient's blood to determine a status of the virus in the patient and additionally treating the patient with the composition if necessary.

Preferably, each of steps (a) and (b) will be repeated at least twice in step (c), and the second amount applied in step (d) will be approximately half of that applied in step (a). Also preferably the treatment will be applied to a patient through injection, and the total amount of the formulation given to the patient in a day will be applied in at least two portions over a period of time.

The treatment method or protocol according to the invention is effective in treating many viruses, and is often effective in completely eradicating the virus in the patient. Where the virus has not been completely eradicated, additional treatment of the virus with the composition can be determined and tailored to the patient to the testing in step (f). The composition according to the invention has mainly stimulatory effects on the phagocytic activity of neutrophils in the human body, and with the initial larger doses provided in steps (a) and (c), the patient often promptly realizes significant relief from the viral infection.

It is an object of the present invention to provide a composition containing peptides and nucleic acids which provides a mainly stimulatory effect, and another such composition which provides a mainly inhibitory effect on phagocystic activity of neutrophils in humans, and which otherwise has substantially no toxicity or ill side effects associated therewith.

It is another object of the invention to provide a relatively simple method of preparing stable solutions of the compositions.

It is a further object of the invention to provide a protocol for treating various viruses in humans using the compositions.

It is yet another object of the invention to provide an improved composition containing peptides and nucleic acid which is tailored or modified to treat different viruses, auto immune diseases and the like.

Other objects, advantages and salient features of the invention will be apparent from the following detailed description which, in conjunction with the annexed drawings, discloses presently preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a photon-counting time series representing the time-resolved phagocyte luminescence of isolated native human neutrophils stimulated with FMLP.

FIG. 2 is a graph depicting a nonmonotonic dependence of the I_p/I_n ratio, employed as a phagocytosis perturbation measure, on the volume (V) of a conventional solution of a composition containing peptides and nucleic acids, where I_n refers to native (i.e., unperturbed) neutrophils, I_p refers to perturbed neutrophils, and I describes the so-called integrated luminescence.

FIG. 3 is a graph showing nonmonotonic changes in the classical perturbation coefficient (CPC) describing the inhibitory effect of the conventional compositions on the phagocytic activity of isolated human neutrophils.

FIG. 4 is a different photon-counting times series $\{n_r(t)\}$ showing the effects on neutrophil phagocytosis induced by a given volume of the conventional composition, in which the descending stage of the $\{n_r(t)\}$ process is described as an exponential decay;

$$n_r(t)=(5512\pm64)\cdot\exp[-(1336\pm7)\times10^{-6}t].$$

FIG. 5 is an absorption spectrum in the UV range of a conventional composition and a corresponding absorption spectrum in the UV range of a composition according to the present invention.

FIG. 6 is a graph showing the volume-dependent modulatory and triggering effect of the composition according to the invention on phagocystosis demonstrated by nonmonotonic changes in the CPC (V) function.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Preparation of the Conventional Composition

According to a known process, the known composition has been prepared over a period of days in the following manner.

First, the indicated quantities of the following components are mixed into ten liters of distilled water under slow stirring:

RAW MATERIALS	AMOUNTS	WEIGHT PERCENTAGE
casein	250 grams	43.9%
blood albumin	15 grams	2.6%
beef peptone	150 grams	26.3%
nucleic acid (RNA)	80 grams	14.0%
sodium hydroxide	75 grams	13.2%

After the ingredients are sufficiently dispersed in the distilled water, the solution is processed under elevated temperatures and pressure using a steam autoclave at a pressure of approximately 5–15 lbs., preferably 8–10 lbs. over a period of approximately 2–10 hours. After the heat and pressure treatment, the solution is then cooled to a temperature somewhat below room temperature and allowed to set until the following day.

The following day the solution is then filtered under an argon gas atmosphere, first through an Ertel asbestos pad filter (# 0.40 or equivalent) after which the pH of the solution is adjusted to approximately 8.5 and the solution is again filtered through a # nine (9) filter pad, after which the solution is then adjusted to pH of approximately 7.8 and again filtered through an Ertel #. EO pyrogen retention filter pad. Again, the filtered solution is refrigerated and stored until the following day. Argon gas is preferred for the gas atmosphere because it is somewhat easier to handle than other gases such as nitrogen, and because its inert nature assures sterility of the resulting composition.

On the third day, the solution is diluted to an appropriate nitrogen content and its pH adjusted to approximately 7.5, after which the solution is passed through a milipore filter HA (0.45μ) for final filtration, after which it is ready for packaging and use.

The conventional composition thus formed is typically stored in sealed glass ampules under Although the example of the known process above uses specific quantities of the respective raw materials, the process may generally use raw materials in the following proportions: 40–50 weight % casein, 1–10 weight % blood albumin, 15–40 weight % beef peptone, 10–25 weight % nucleic acid, and 5–25 weight % base. an argon gas atmosphere, in appropriate quantities such as 2.5 ml and 10 ml vials.

Preparation of the Modified Composition of the Invention

The composition according to the invention is similar to the conventional composition except that it is additionally or more specifically filtered or centrifuged to remove or separate lower molecular weight active components. The composition according to the invention may be obtained by further processing the conventional composition.

According to the present invention, the conventional composition is preferably further processed by a dialysis thereof through a semi-permeable membrane having an average pore radius of approximately 10–40 Angstroms, and most preferably having an average pore radius of 24 Angstroms. Alternatively, the further processing of the conventional composition could be effected using appropriate centrifugation techniques. Appropriate semipermeable membranes or dialysis tubing may be acquired from Viskin of Serva Germany. The threshhold for molecular weight (MW) of molecules removed or separated by dialysis according to the invention is in the range of 8–15 kDa, and most preferably all molecules with a MW of <15 kDa. Applicant has discovered that the heavier active components remaining in the modified or dialyzed composition according to the invention, MW≧15–25 kDa and including peptides without aromatic components, stimulate phagocytosis of the neutrophils in humans when applied above a certain quantity thereof; and that the smaller active components removed from the composition through dialysis, including small nucleic acid fragments associated with peptides containing aromatic amino acids and having molecular weights in the range of approximately 1–15 kDa, normally function as phagocytosis inhibitors at all concentrations thereof. Additionally applicant has discovered that the conventional composition exhibits a phenomenon of modulation of the neutrophil phagocytic activity caused by the interplay between the lower molecular weight components (<8–15 kDa) and the larger components (≧8–15 kDa, i.e., the smaller components inhibit the stimulatory effect of the larger components. Correspondingly, in the modified composition according to the invention the smaller active components are removed so that the full stimulatory effect of the larger active components is achieved, while the smaller active components may be used in treating auto immune diseases.

Moreover, even within the range of larger, stimulating active components, applicant has been able to isolate narrower ranges of the active components which are more effective in treating different viruses, such as HIV, influenza, herpes, etc.

The much enhanced effectiveness of the composition according to the invention as an antiviral agent is understood from the following in vitro tests conducted by applicant comparing the composition according to the invention with the conventional composition. Measurements in the testing procedure were made using a single photon-counting method in order to record the time-resolved phagocyte luminescence or chemiluminescence of human isolated neutrophils. Such luminescense was first observed in 1972 and has been used since as an effective measurement of phagocytic activity and the like. Allen R. C. Stjernholm R. L., and Stele R. H., Evidence Of The Generation of (An) Electronic Excitation State(s) in Human Polymorphonuclear Lukocytes And Its Participation in Bacterial Activity, *Biochem. Biophys. Res. Commun.*, 47, 679–684, 1972. The phagocyte luminescence, a phenomenon, involves reaction of certain products of oxygen reduction generated by stimulated neutrophils or other cells. As discussed above, it is believed that the conventional composition and the modified composition according to the invention enhance the leukocytic response, increase antibody production and stimulate phagocytosis of human neutrophils, and the inventor sought to verify this thesis of phagocytic function of neurophils by means of a single photon-counting technique applied to a time-resolved phagocytic luminescence of isolated human neutrophils incubated with the composition and then stimulated with FMLP. Applicant's testing, as described fully below, not only verifies the thesis, but shows that the composition according to the invention functions as a potent stimulator of phagocytosis in human neutrophils.

Material and Method

Experimental material consisted of human neutrophils obtained from venous blood of fifteen adult healthy subjects, then isolated according to Böyum's method. See Böyum A; Isolation of Lymphocites, Granulocytes and Macrophages, *Scand. J Immunol.* 5 (Supp 5), 9–15, 1976. The cells were counted in a Bürker's chamber and their types were determined by a Pappenheim staining procedure. The cell samples contain over 90 percent mature neutrophils, their viability evaluated by a trypan blue (1% solution in 0.15M NaCl) exceeded 95 percent.

A standard buffer solution (SBS), commonly used in chemiluminescent research was composed of phosphate buffered saline (pH 7.4), 10mM glucose and 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] available from Calbiochemin Switzerland.

The cell samples contain 3×10^5 neutrophils in 3 ml of SBS, were incubated at 37° C. for a period of 5 minutes with 5 microliters of the conventional composition or of the composition according to the invention in different concentrations. After the 5 minute incubation luminol (a 2.5 μM final concentration), available from Koch-Light Lab in England, was added to the samples. After the next 5 minutes phagocytic processes were initiated by FMLP, available from Sigma Chemical in the United States, then chemiluminescent processes were registered at a temperature of 37° C. by means of a single photon counting technique using a M12FQ51 photoamplifier.

Solutions of the composition according to the invention as used in the testing were made by dialyzing the conventional composition as discussed above, and particularly by dialyzing a 4 ml quantity of the conventional composition to one liter of physiological solution during 48 hours at 4° C., and the resulting solution again consisted essentially of active components of the compositions having a molecular weight in the range of 15–25 kDa.

Given the diluted nature of the dialyzed solution, and in order to redress the absorbance of A=0.77 in the 200–235 nm band which had been caused by a given volume (V) of the conventional composition, it was necessary to use the dialyzed solution of the invention at a volume 12 times as large as the volume (V) of conventional composition used. The larger volume of dialyzed composition was used in compiling data for FIG. 5.

The chemiluminscent processes have been recorded in form of photon-counting time series $[n(t):t=1,2, \ldots ,N]$, composed of the numbers of photoelectrons n(t) registered in consecutive time intervals $(t,t+\Delta t_c)$, with a counting time $\Delta t_c=1$ s, separated by the same length intervals (constituting the dead time interval of recorder, $\Delta t_d=\Delta t_c$ during which no photoelectrons were registered). The quantity n(t) is proportional to the number of photons emitted by the light producing-system and submitted to the same statistics. An example of photon-counting time series (PCTS) describing the emission from aative neutrophils stimulated by FMPL is shown in FIG. 1. Analogous PCTS, in respect to shape but not of the magnitude, occur for neutrophils incubated with the conventional composition or with the composition according to the invention.

RESULTS AND DISCUSSION

Effects of Conventional Composition on Phagocytosis

An integrated intensity of emission $I=\Sigma_t n(t)$ was measured in a time interval [1,N], corresponding to a whole process (composed of ascending and descending stages). Surprisingly, the measurements show that the samples of neutrophils treated with the conventional PNA formulation composition had integrated intensities (I_p) lower than those (I_n) of native or untreated neutrophils samples. In other words, a perturbation of phagocytosis, reflected by the inequality $I_p<I_n$ and corresponding to an inhibition of phagocytic activity of neutrophils, was demonstrated by the samples treated with the conventional composition.

The effect of the conventional composition on a phagocytic activity of neutropils was determined using the raio I_p/I_n(again refer to the Allen article discussed above) and a perturbation coefficient $CPC=(1-I_p/I_n)\cdot100[\%]$, where the perturbation coefficient is normalized to 100% and directly proportional to the magnitude of perturbation or inhibition. See Kochel B., Time-Resolve Luminescence of Perturbed Biosystems: Scholastic Models and Perturbation Measures, *Experimentia*, 48, 1059–1069, 1992. The experimental results of the samples treated with a conventional Reticulose™ formulation are shown in Table 1.

TABLE 1

Reticulose volume per the sample*) V [μl]	Ratio of the integrated intensities I_p/I_n	Perturbation measure CPC ± SD(CPC)**) [%]
0.063	0.388	61.2 ± 4.9
0.063	0.560	44.0 ± 4.2
0.078	0.567	43.3 ± 4.0
0.083	0.466	53.4 ± 3.7
0.100	0.480	52.0 ± 4.5
0.125	0.386	61.4 ± 4.8
0.156	0.695	30.5 ± 3.8
0.167	0.734	26.6 ± 4.2
0.167	0.664	33.6 ± 3.3
0.200	0.426	57.4 ± 4.0
0.250	0.422	57.8 ± 4.6
0.250	0.401	59.9 ± 4.7
0.313	0.337	66.3 ± 4.1
0.500	0.402	59.8 ± 3.6
0.500	0.329	67.1 ± 3.6
0.625	0.188	81.2 ± 3.9
1.000	0.248	75.2 ± 4.1
1.000	0.223	77.7 ± 3.4
1.250	0.253	74.7 ± 4.3
1.250	0.228	77.2 ± 3.7
1.250	0.224	77.6 ± 4.5
2.500	0.113	88.7 ± 3.8
5.000	0.128	87.2 ± 4.4

*)Sample volume: 3 ml.
**) $SD(CPC) = 10^4 \cdot N/I_n \cdot [1 + (I_p/I_n)^2]^{1/2}$ at SD(n(t)) = 100 cps.

The volume V of the conventional composition has been used as a control variable for the experiment, and a corresponding concentration of the composition in each sample (3ml) is expressed by the formula c[volume percentage]=V [μl]/30. In the interval [0.063, 0.139] μl a linear regression of the ratio I_p/I_n on V,

$$I_p/I_n=(2.04\pm0.82)\cdot V+0.32\pm0.10) \quad (1)$$

is the best one (r=0.69±0.18) amongst other elementary regressions such as power (r=0.59±0.22), logarithmic (r=0.63±0.20) or exponential (r=0.64±0.20). Refer to FIG. 2 for the linear regression.

From Eq.1 and the definition of CPC above, the following dependence of CPC on V results:

$$CPC=\{1-[(2.04\pm0.82)\cdot V+(0.32\pm0.10)]\}\cdot100 \quad (2)$$

The coefficients in all the regression equations are expressed together with their standard deviations (SD).

At the volumes V>0.139 μl a power regression of I_p/I_n on V (Table I, FIG. 2),

$$I_p/I_n(V)=(0.23\pm0.02)\cdot V^{-}(0.49\pm0.05) \quad (3)$$

fits better (r=0.93±0.04) the experimental data points than logarithmic (r=0.87±0.06) or exponential (r=0.76±0.11) ones. Therefore a CPC (V) function takes the form:

$$CPC(V)=[1-(0.23\pm0.02)\cdot V^{-}(0.49\pm0.05)]\cdot 100. \quad (4)$$

From Eqs. 2 and 4 it can be seen that an inhibitory effect of the conventional composition on a phagocytic activity of neutrophils decreases when the conventional composition volume V tends from 0.063–0.139 μl then the inhibitory effect increases with V at V≧0.139 μl as shown in FIG. 3.

The results obtained using the conventional composition indicate an inhibition of phagocytosis in the entire volume range tested. Nonmontonic changes, similar to those induced by the conventional composition in the inhibition of phagocytosis, observed in the CPC=CPC(V) function (FIG. 3), are also known, although unexplained, in chemiluminescence of neutrophils in the presence of plasma. Faden H., Luminol-Dependent Whole Blood Chemiluminescence Assay, *Cellular Chemiluminescence*, V. 11 K. Van Dyke and V. Castranova (Eds.), CRC Press Boca Raton 1987, pp 183–191.

Additionally, it should be noted that the inhibitory effect of the conventional composition and the neutrophil phagocytosis does not depend on the order of addition of the convention composition and FMLP (activator). This means that the inhibition is neither caused by the receptor-ligand interaction nor by the signal transduction to the cell. This fact and a good fitting (r=0.997±0.001) shown in FIG. 4 of the $[n_r(t)]$ series by an exponential regression.

$$n_r(t)=(5512\pm64)\cdot\exp[-(1336\pm7)\times10^{-6}\cdot t], \quad (5)$$

where $\{n_r(t)\}$ is a difference series resulting from the $\{n_n(t)\}$ series (the phagocyte luminescence of native neutrophils) by substracting the $\{n_p(t)\}$ series (the phagocyte luminescence of neutrophils perturbed with 0.25 μl of the conventional composition) appear to support a possibility of scavenging of oxygen radicals by peptide nucleic acids. At k=1336 and $\Delta t_c=\Delta t_r=1$ s the solution in Eq. 5 corresponds to a first-order (α=1) reaction with the rate constant $k_r=k\cdot\Delta t_c^{\alpha-1}\cdot 2^{-\alpha}=668$ photocount/s.

By comparing the results obtained for these samples using the conventional composition with those discussed below obtained using the improved composition according to the present invention, one can conclude that the inhibition obtained using the conventional composition is caused by low-molecular weight formulations (MW<8–15kDa) absorbing at 235–300 nm in the UV spectrum. These compositions have already been identified as nucleic acid fragments and/or nucleic acids associated with peptides. W. N. Strickland, Summary of Peptide Nucleic Acid Studies conducted at the University of Wisconsin Biotechnology Center, *Reticulose, Commonwealth Pharmaceuticals, Trenton*, 1995, pp. 19–35. One possible explanation of the inhibition can be based on the influence of these "small" molecules on the final stage of phagocytosis, i.e., they can play a role of scavengers of oxygen radicals. Such a phenomenon is known for, e.g., plasma where endogenous inhibitors of oxygen radicals quench luminescence. See the section by H. Faden in Cellular Chemiluminscent discussed above. Another alternative, and not necessarily disjunctive, explanation can be related to the influence of the conventional composition on the early stages of phagocytosis, e.g., the receptor expression, certain metabolic pathways, etc.

Effects of the Improved Composition of the Invention on Phagocytosis

As discussed above, the improved composition according to the invention contains active components/molecules with molecular weight greater than 8–15 kDa, preferably in a range of 15–25 kDa, and which are characterized by an absorption band (Δλ=200–235 nm, λ^{max}=205 nm, A=0.06) in the UV spectrum as shown in FIG. 5. By comparison, a sample of the conventional composition at the same absorption band (200–235nm) has an absorption of A=0.77. In order to redress such absorbance of the conventional composition in the samples involving the improved composition according to the invention, it is necessary to use the improved composition in a volume of $V_D=12\cdot V$, where V is a given volume of the conventional composition. Results of the tests involving the improved composition according to the invention are set forth in Table 2 below.

TABLE 2

Equivalent Reticulose volume *) per the sample **) V [μl]	Ratio of the integrated intensities I_p/I_n	Perturbation measure CPC ± SD(CPC) [%]	Remarks
0.167	0.648	35.2 ± 5.3	Inhibition of phagocytic activity of neutrophils
0.420	0.476	52.4 ± 4.4	
0.830	0.452	54.8 ± 2.4	
0.830	0.436	56.4 ± 3.7	
1.250	0.292	70.8 ± 9.7	
2.500	0.751	24.9 ± 3.7	
2.500	0.925	7.5 ± 4.5	
2.500	1.051	−5.1 ± 4.7	Stimulation of phagocytic activity of neutrophils
5.000	1.220	−22.0 ± 4.6	
5.000	1.359	−35.9 ± 4.8	
10.000	1.512	−51.2 ± 8.3	
10.000	1.974	−97.4 ± 4.4	
20.000	3.454	−254.4 ± 9.2	

*) A given volume (V_D) of the Reticulose dialysate has been expressed by the equivalent volume of Reticulose (V) causing the same absorbance at 205 nm.
**) Sample volume; 3 ml.

Generally speaking, CPC>0 at $I_p<I_n$, whereas if $I_p>I_n$ then CPC<0 and therefore a quantity CPC_+ defined by the equation $CPC_+=(I_p/I_n-1)\cdot100$ describes a stimulation of phagocytosis in percentages.

With reference to Table 2, in the volume interval [0.167, 0.984]μl interval the changes in the I_p/I_n or CPC values (as shown in FIG. 6), caused by changes in V (cf.Table 2) are expressed by regressions:

$$I_p/I_n(V)=1-(0.063\pm(0.03)\cdot V^{(0.31\pm0.06)}, \quad (6)$$

$$CPC\ (V)=(62.6\pm2.0)\cdot V(0.31\pm0.06), \quad (7)$$

at a correlation coefficient r=0.96±0.04. Therefore, in the discussed volume interval the inhibition of phagocytosis, caused by the improved composition of the invention, increases with the volume of the formulation used.

According to equations 4 and 7 the inhibition caused by the conventional composition at V=[0.167, 0.984] μl is higher than that by the composition of the invention by 8.8–17.2 percentage points. This means that the redress of the concentration of active components in the composition of the invention, which absorb in the 200–235 nm interval, is not sufficient for redressing the perturbation of phagocytosis to the level caused by conventional composition. Such 8.8–17.2 percentage point decrease in the perturbation is directly related to the absence of those smaller molecular weight formulations (MW<8–15 kDa) which were removed or dialyzed away according to the invention. Since those

smaller molecular weight formulations have an absorption band at 235–300 nm one can conclude that they are nucleic acids and/or their associations with peptides, and state that their contribution to the inhibition of phagocytosis ranges from 8.8 to 17.2%. Consequently the higher molecular weight formulations (MW >8–15 kDa) occuring in the composition according to the invention show an 82.8–91.2 percent contribution to the inhibition phagocytosis in the [0.167, 0.984] μl range.

In a higher range [0.984, 3.764] μl interval the changes in I_v/I_n or CPC (FIG. 6), caused by changes in V (cf. Table 2), are expressed by the regressions:

$$I_v/I_n(V)=(0.38\pm0.07)\cdot V^{(0.73\pm0.11)} \quad (8)$$

$$CPC(V)=[1-(0.38\pm0.07)\cdot V^{(0.73\pm0.11)}]\cdot 100, \quad (9)$$

at a correlation coefficient r=0.94±0.04. There occurs an inhibition of phagocytosis caused by the composition of the invention at V=[0.984,3.764] μl and a stimulation at V>3.764 μl because CPC(3.764)=0.

The composition, according to the invention, inhibits a phagocytosis at V=[0.984,3.764] μl, although the inhibition decreases to zero, according to Eq. 9, when V increases to 3.764 μl. In comparison with the inhibition caused by the conventional composition, there is discerned a difference ranging from 14.4 to 88.0 percentage points. For instance, 3.764, μl of the composition of the invention does not inhibit a phagocytosis (CPC=0) whereas the same volume of the conventional composition inhibits phagocytosis to 88.0 percentage points. Again, complexes of peptides and nucleic acids appear to be responsible for that inhibition.

The difference between the composition of the invention in comparison to the conventional composition are particularly noticeable at volumes greater than 3.764 μl because the formulation of the invention stimulates a phagocytosis at such volumes, as represented by negative values of CPC in Eq. 8 above, whereas the conventional composition continues to inhibit the phagocytosis as reflected by the sample volume of 5.000 μl in Table 1 above showing that the conventional formulation inhibited phagocytosis at 87.2 percentage points.

Whereas negative values of CPC in Eq. 8 above indicate stimulation of phagocytosis, positive values of the CPC (V) function in Eq. 10 below correspondingly indicate stimulation of phagocytosis.

$$CPC_+(V)=[(0.38\pm0.07)\cdot V^{(0.73\pm0.11)}-1]\cdot 100 \quad (10)$$

It should be emphasized that the composition according to the invention did not change the phagocytic activity of neutrophils (CPC=0) after the neutrophils have been stimulated by FMLP, i.e., the changes in the phagocytic activity were observed only when neutrophils have been treated by the composition prior to the stimulation by FMLP. For instance, the 245% stimulaton of phagocytosis occuring at V=20 μl was possible only when the incubation with the composition preceded the addition of FMLP. This fact indicates the phenomenon of priming. Downey G. P., Fukushima T., Fialkow L. and Waddell T. K., Intracellular Signalling In Neutrophil Priming and Activation, *Sem. In Cell Biol.*, 6, 345–356, 1995. The priming is caused by the active components in the composition with MW>8–15 kDa, because that phenomenon does not occur for the conventional Reticulose™ formulation where it is blocked by the low molecular-weight active components (MW <8–15 kDa), i.e., nucleic acid fragments and their associations with peptides.

Amongst the possible pathways by which the formulations contained in the composition of the invention affect neutrophil phagocytosis, the following are most likely: the influence on a receptor expression; binding of a stimulus (FMLP) with receptors; transduction of a signal to the cell interior; and the activation of NADPH-oxidase. The above-mentioned and other forms of influence on metabollic pathways result in some changes in the production of active forms of oxygen (e.g., $^1O_1$,O_2^-) which in turn determine the final stage of phagocytosis.

Conclusions

As understood from the foregoing test results, the conventional PNA formulation inhibits a phagocytic activity of neutrophils for all of the investigated volumes. The inhibition decreases linearly (CPC(V)=2.04·V+0.32) in the [0.063, 0.139] μl interval, then increases nonlinearly ($CPC(V)=[1-0.23\cdot V^{-0.49}]\cdot 100$) at V>0.139 μl with the conventional composition used. The inhibitory effect of the conventional composition on phagocytosis of neutrophils is caused by nucleic acid fragments (possibly associated with peptides) having a low molecular weight, i.e., MW<8–15 kDa, whereas the composition according to the invention with active components having MW >8–15 kDa stimulate phagocytosis.

Specifically, the composition of the invention inhibits a phagocytic function of isolated human neutrophils at low volumes (<3.674 μl) of the diluted solution used. The inhibition of phagocytosis increases according to a power low ($CPC(V)-62.6\cdot V^{0.31}$) for volumes below 0.984 μl and then decreases nonlinearly ($CPC(V)=[1-0.38\cdot V^{0.73}]\cdot 100$) for V>0.984 μl. At the volume threshold of 3.764 μl it causes no inhibition of a phagocytic function of neutrophils, and above such volume threshold it stimulates such phagocytic function. The stimulation changes with the composition volume according to a power low ($CPC_+(V)=[0.38\cdot V^{0.73}-1]\cdot 100$). Reactive components of the composition according to the invention (peptides without aromatic components) characterized by MW>8–15 kDa and absorption at 200–235 nm, play a role of priming factors, which convert neutrophils to a status more "respondent" to external stimuli such as FMLP.

The presence of peptide nucleic acids with MW<8–15 kDa (as it holds for the conventional composition) annihilates the stimulation of phagocytosis by the heavier (MW>8–15 kDa) active components. On the other hand, the compositions according to the invention, having mainly stimulatory effects on a phagocytic activity of neutrophils, are obtained by separating away the small components (MW<8–15 kDa) by a dialysis or centrifuge of the conventional composition through an appropriate semipermeable membrane, such as a 24 Angstrom membrane or through appropriate centrifugation. Further, the removed or separated lower weight active components (MW<8–15 kDa) can be used in treating auto immune diseases as discussed above. Basically, the lower weight active components of the composition function to slow down the degeneration caused by the auto immune diseases. Moreover, the composition according to the invention can be tailored to treat different viruses by further narrowing a range of molecular weights of the active components contained therein.

Use of the Composition of the Invention

The compositions of the present invention are most preferably administered by way of injectable aqueous solutions or preparations, discussed further hereinbelow in relation to specific examples of uses, but may be otherwise administered orally, parenterally, sublingually, by inhalation spray,

rectally, or topically in dosage unit formulations containing appropriate non-toxic carriers, adjuvants and vehicles as desired. The term parenteral encompasses subquetaneous injections, intravenous, intramuscular, intraternal injection or infusion techniques.

As an injectable aqueous solution, the composition according to the invention may be packaged in appropriate sized glass ampules similar to manner in which the conventional composition is packaged as discussed above, or in appropriate larger stoppered vials.

According to the invention, different protocols have been developed for treating different viral infections with the compositions of the invention. Below are presented four protocols for treating herpes/genital warts; Hepatitis B; Hepatitis C. Chronic Fatigue, Epstein-Barr; and HIV using injectable aqueous solutions of the compositions of the invention. According to an important aspect of the invention, as discussed above, the composition may be modified or specifically adapted for treating different viruses.

Protocol No. 1

Have test performed for Herpes A.B and C. include IGG, IGA.

Inject composition solution twice daily for three (3) days—1 mL each injection.

Take no medication for seven (7) days.

Inject composition Solution twice daily for three (3) days—1 mL each injection.

Take no medication for seven (7) days.

Inject composition solution twice daily for three (3) days—1 mL each injection.

Wait 14 days.

Have Herpes test performed for specific Herpes type established in original Herpes panel (have both IGG and IGA performed).

The composition solution may initially be refrigerated, though it need not be. If so, bring the syringe to body temperature before injection (e.g. holding it in hand). Inject slowly to provide painless infusion. If there is pain at injection site because of cold composition or too fast injection, pain will dissipate within 15 minutes. Rotate injection sites. NOTE: 60% Of Herpes patients clear infection using this protocol. The remaining 40% are persons that have a weakened immune system because of stress or other causes such as sunburn. For these patients, it is recommended to provide a maintenance dosage of ½ mL twice a day for one day per month to prevent recurrence.

Protocol No. 2

Hepatitis B

Have test performed for Hepatitis B include IGG, IGA.

Inject composition solution—1 mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—1 mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—1 mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—½ mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—½ mL two times daily for 7 days. Next 7 days—nothing.

Do respective blood work 2 months after protocol has been completed, and adjust per attending physician's recommendation.

Rotate injection sites.

Hepatitis C. Chronic Fatigue Epstein-Barr

Inject composition solution—1 mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—1 mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—1 mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—½ mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—½ mL two times daily for 7 days. Next 7 days—nothing.

Do respective blood work after protocol has been completed, and adjust per attending physician's recommendation.

Vitamin C I.V. should be given in conjunction with subcutaneous medication for a minimum of 28 days.

20 grams Ascorbic Acid	10 mL Calcium Gluconate
6 mL Magnesium Chloride	1 mL B-Composition
1 mL B-12	1 mL B-6
Adjust pH to 7.4 with Sodium Bicardonate	

Put above ingredients in 500 mL sterile distilled water and run for 3 hours daily for no less than 28 days. After each 7 days add additional 10 grams of Ascorbic Acid to a maximum of 50 grams Ascorbic Acid in the last week of Vitamin C I.V., treatments as long as patient can tolerate additional Vitamin C. If patient develops diarrhea or upset stomach back off Vitamin C (Ascorbic Acid to previous tolerable level).

Protocol No. 4 HIV

Inject composition solution—1 mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—1 mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—1 mL two times daily for 7 days. Next 7 days—nothing.

Inject composition solution—½ mL two times daily for 7 days. Next 7 days—nothing.

Do respective blood work including P-24 antigen test 2 months after protocol has been completed, and adjust per attending physician's recommendation.

Rotate injection sites.

These four exemplary protocols according to the invention reflect the effectiveness of the improved composition in treating many viruses, and auto immune diseases and often are effective in completely eradicating the virus or disease in the patient. Where the virus or disease is not completely eradicated, additional treatment of the virus with the composition according to the invention can be determined and tailored to the patient through testing of the patient after administration of the protocol program. With the initial larger doses provided in the early steps according to the protocols of the invention, patients often promptly realize significant relief from the viruses and diseases.

Although there have been described above what are considered to be presently preferred embodiments of the invention, it will be understood as various changes and modifications may be made thereto without departing from the spirit or essence of the invention. The scope of the

invention is indicated by the appended claims, rather than by the foregoing description.

I claim:

1. In a composition containing peptides and nucleic acids, with components having molecular weights in a range from about 1–25 kDa and which absorbs light in at least two bands having maximum values at 205 nm and 260 nm, and wherein the composition is initially formulated by processing a mixture of casein, blood albumin, beef peptone, nucleic acid, and a base in an appropriate medium at an elevated temperature and an elevated pressure, and separating therefrom components having molecular weights outside of the range from about 1–25 kDa, the improvement comprising:

processing said composition to remove therefrom components having molecular weights of <8 kDa such that the processed composition absorbs light in a wavelength interval from about 200–235 nm with a maximum absorbance at 205 nm.

2. The composition according to claim 1, wherein said composition is processed by dialyzing the composition through a membrane having an average pore size of 10–40 Angstroms.

3. The composition according to claim 1, wherein said mixture contains 40–50 weight % casein, 1–10 weight % blood albumin, 1–3 weight % beef peptone, 10–25 weight % nucleic acid, and 5–25 weight % base.

4. The composition according to claim 1, wherein said mixture contains 43.9 weight % casein, 2.6 weight % blood albumin, 26.3 weight % beef peptone, 14.0 weight % nucleic acid, and 13.2 weight % sodium hydroxide.

5. In a composition containing peptides and nucleic acids, with components having molecular weights in a range from about 1–25 kDa and which absorbs light in at least two bands having maximum values at 205 nm and 260 nm, and wherein the composition is initially formulated by processing a mixture of casein, blood albumin, beef peptone, nucleic acid, and a base in an appropriate medium at an elevated temperature and an elevated pressure, and separating therefrom components having molecular weights outside of the range from about 1–25 kDa, the improvement comprising:

processing said composition to remove therefrom components having molecular weights of ≧8 kDa such that the processed composition absorbs light in a wavelength interval from about 235–300 nm.

6. The composition according to claim 5, wherein said composition is processed by dialyzing the composition through a membrane having an average pore size of 10–40 Angstroms.

7. The composition according to claim 5, wherein said mixture contains 40–50 weight % casein, 1–10 weight % blood albumin, 1–3 weight % beef peptone, 10–25 weight % nucleic acid, and 5–25 weight % base.

8. The composition according to claim 5, wherein said mixture contains 43.9 weight % casein, 2.6 weight % blood albumin, 26.3 weight % beef peptone, 14.0 weight % nucleic acid, and 13.2 weight % sodium hydroxide.

9. In a method of preparing a composition containing peptides and nucleic acids comprising the steps of:

forming a mixture of casein, blood albumin, beef peptone, nucleic acid and a base in an appropriate medium;

processing the mixture at an elevated temperature and an elevated pressure;

separating from the processed mixture components having molecular weights outside of a range from about 1–25 kDa such that the mixture absorbs light in at least two bands having maximum values at 205 nm and 260 nm;

the improvement comprising the step of:

further removing from the mixture components having molecular weights of <8 kDa such that the mixture absorbs light in a wavelength interval from about 200–240 nm with a maximum absorbance at 205 nm.

10. The method according to claim 9, wherein said further separating step involves dialyzing the mixture through a membrane having an average pore size of 10–40 Angstroms.

11. The method according to claim 9, wherein said mixture contains 40–50 weight % casein, 1–10 weight % blood albumin, 1–3 weight % beef peptone, 10–25 weight % nucleic acid, and 5–25 weight % base.

12. The method according to claim 9, wherein said mixture contains 43.9 weight % casein, 2.6 weight % blood albumin, 26.3 weight % beef peptone, 14.0 weight % nucleic acid, and 13.2 weight % sodium hydroxide.

13. In a method of preparing a composition containing peptides and nucleic acids comprising the steps of:

forming a mixture of casein, blood albumin, beef peptone, nucleic acid and a base in an appropriate medium;

processing the mixture at an elevated temperature and an elevated pressure;

separating from the processed mixture components having molecular weights outside of a range from about 1–25 kDa such that the mixture absorbs light in at least two bands having maximum values at 205 nm and 260 nm;

the improvement comprising the step of:

further removing from the mixture components having molecular weights of >8 kDa such that the mixture absorbs light in a wavelength interval from about 235–300 nm.

14. The method according to claim 13, wherein said further separating step involves dialyzing the mixture through a membrane having an average pore size of 10–40 Angstroms.

15. The method according to claim 13, wherein said mixture contains 40–50 weight % casein, 1–10 weight % blood albumin, 1–3 weight % beef peptone, 10–25 weight % nucleic acid, and 5–25 weight % base.

16. The method according to claim 13, wherein said mixture contains 43.9 weight % casein, 2.6 weight % blood albumin, 26.3 weight % beef peptone, 14.0 weight %.

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US006303153B1

(12) **United States Patent**
Friedland et al.

(10) **Patent No.: US 6,303,153 B1**
(45) **Date of Patent: Oct. 16, 2001**

(54) **PREPARATION OF A THERAPEUTIC COMPOSITION**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/344,095**

(22) Filed: **Jun. 25, 1999**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/735,236, filed on Oct. 22, 1996, now abandoned.

(51) **Int. Cl.**^{7} **A61K 38/01**

(52) **U.S. Cl.** **424/529**; 424/535; 424/548; 424/195.1; 514/2; 514/21; 514/44

(58) **Field of Search** 424/535, 529, 424/195.1, 548; 514/2, 21, 44

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Primary Examiner—Jean C. Witz
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(57) **ABSTRACT**

Product R, a novel therapeutic composition for treating viral infections and stimulating the immune system, comprises nucleotides and peptides that have molecular weights not more than 14 KDa and substantially not more than 8 KDa. The composition has a light absorption spectrum with typical absorption ratios of 1.998 at 260 nm/280 nm and 1.359 at 260 nm/230 nm.

11 Claims, 8 Drawing Sheets

(2 of 8 Drawing Sheet(s) Filed in Color)

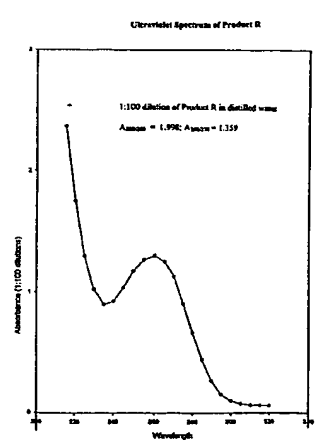

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Figure 1. Ultraviolet Spectrum of Product R

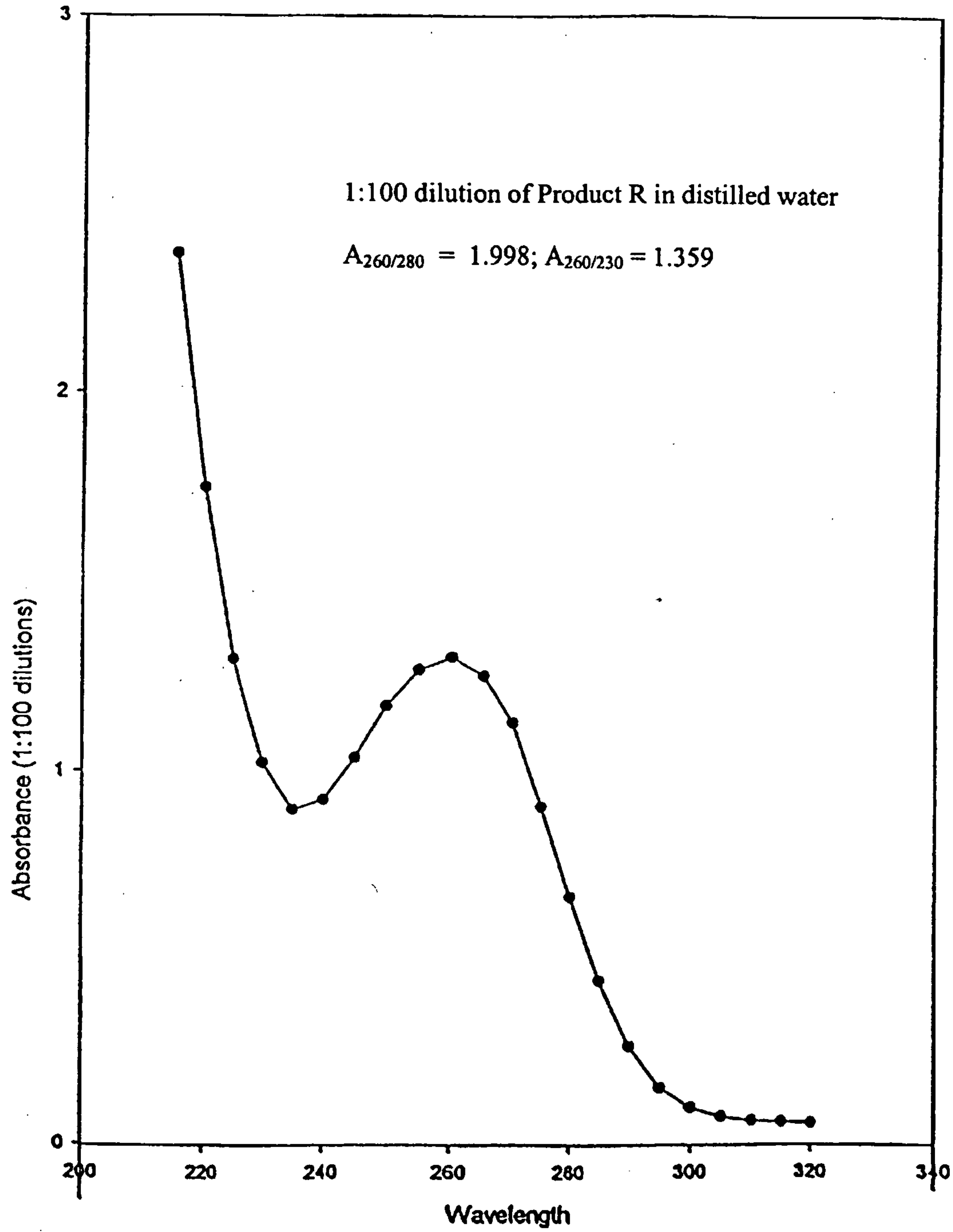


Figure 2. HPLC Chromatogram of Product R

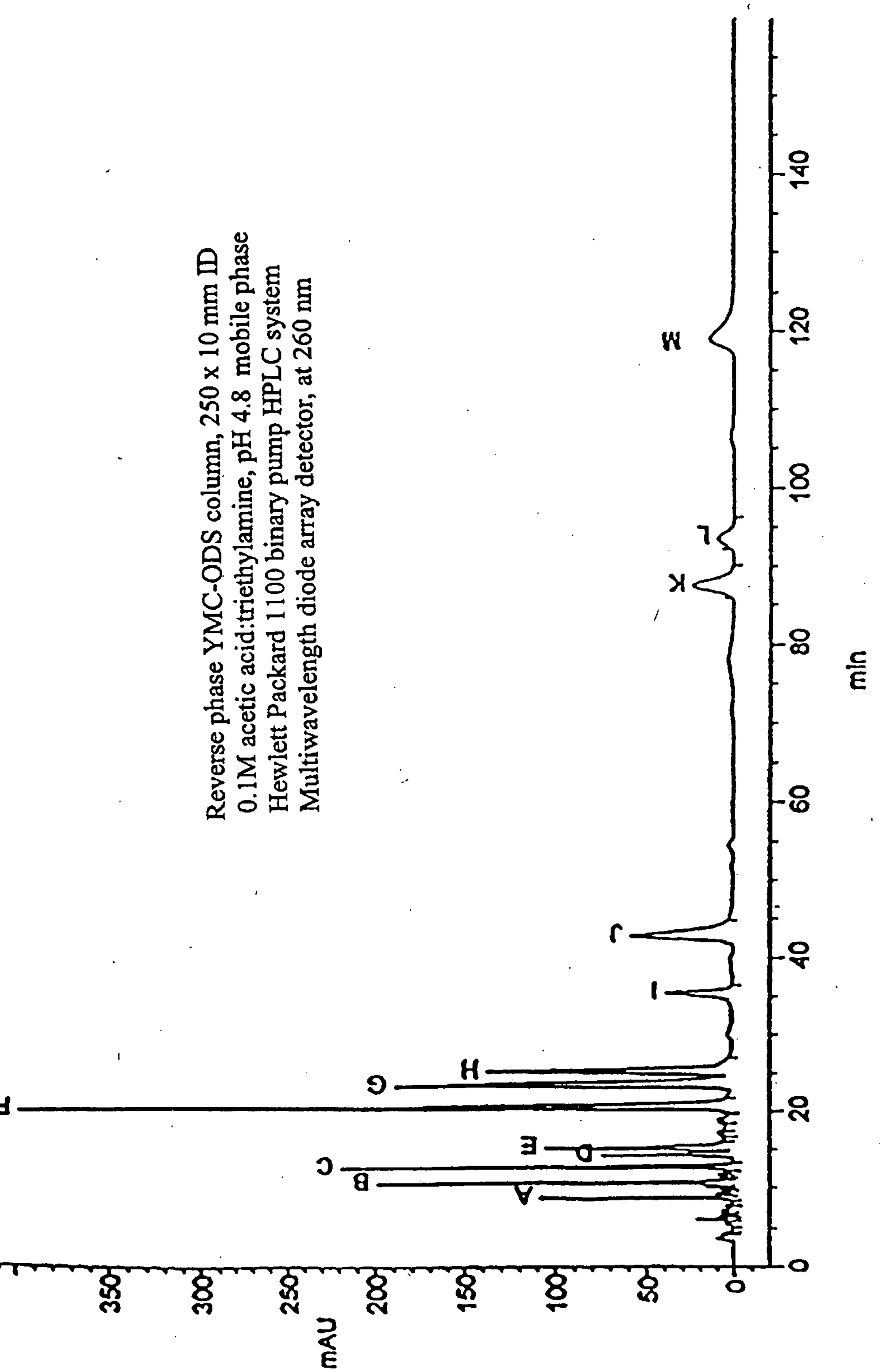
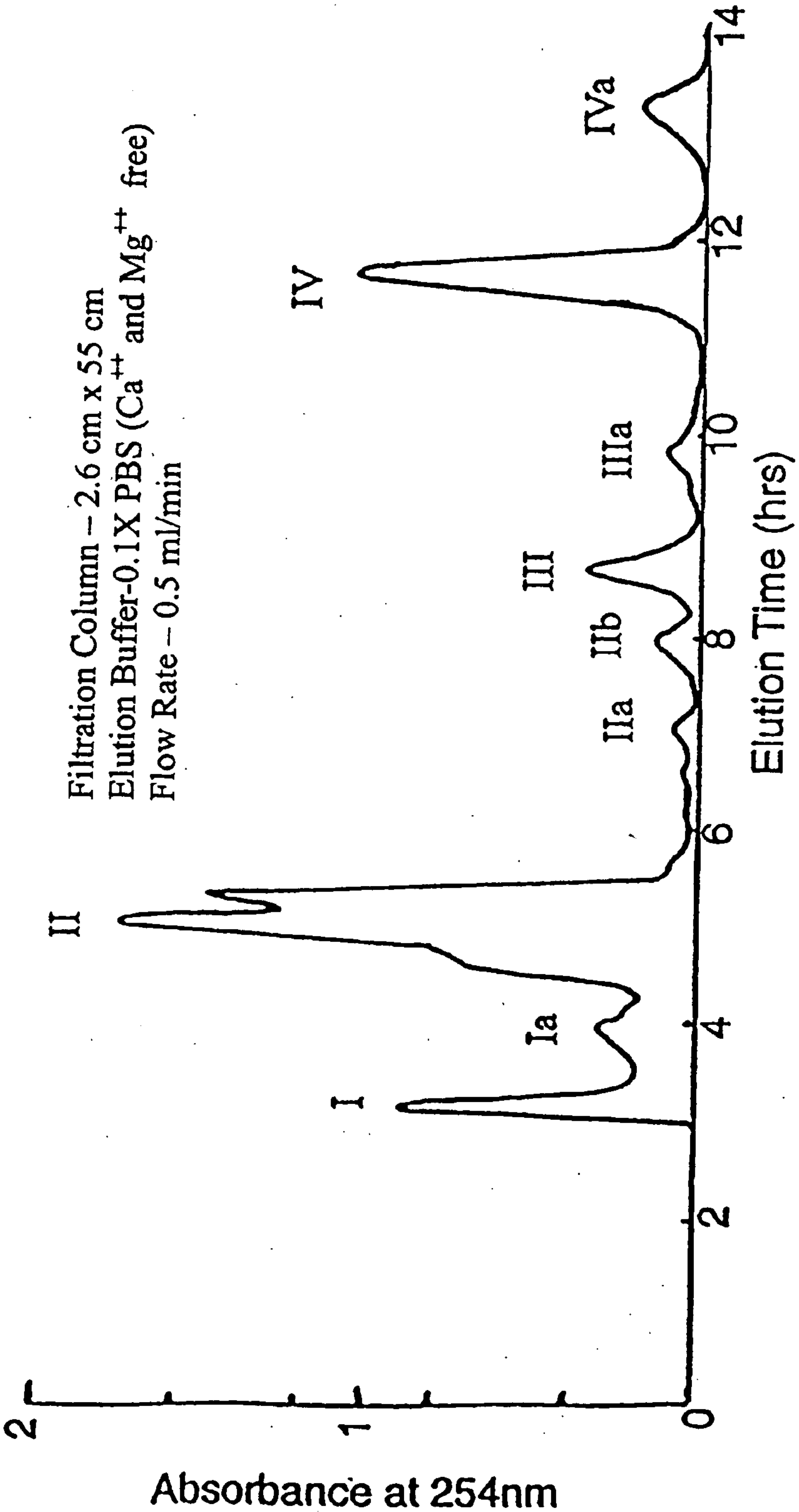


Figure 3. Fractionation of Product R by BioGel P-2 Column



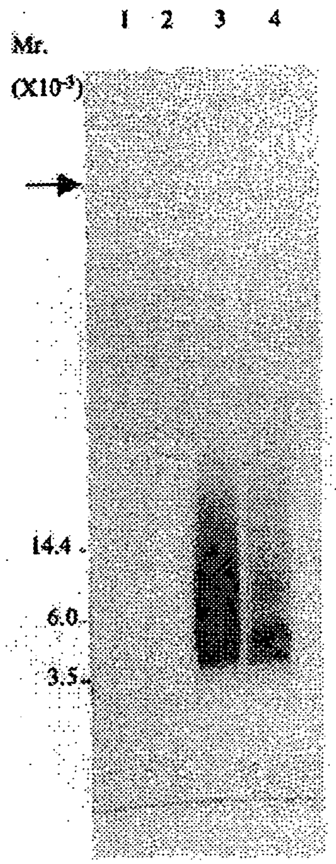
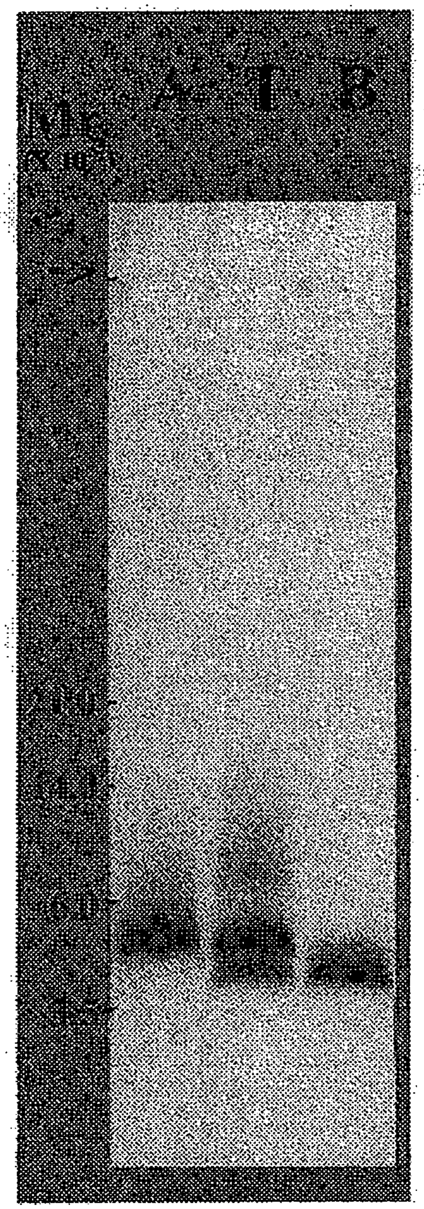


Figure 4. SDS-Polyacrylamide Gel Electrophoresis of Product R and Fractions

Product R and Peak I fractions show two strong silver stained bands . Later fractions show no band.

Fractions were ten-fold concentrated; 2 ul of Product R and 1 ul of concentrated fractions were used.

Lane 1: Concentrated Peak II; Lane 2: Concentrated Peak Ia; Lane 3: Concentrated Peak I; Lane 4: Product R. Arrow indicates the top of the lane.



A-Fraction A
T-Total Product R
B-Fraction B

Figure 5. SDS-PAGE Profile of Product R Peptides

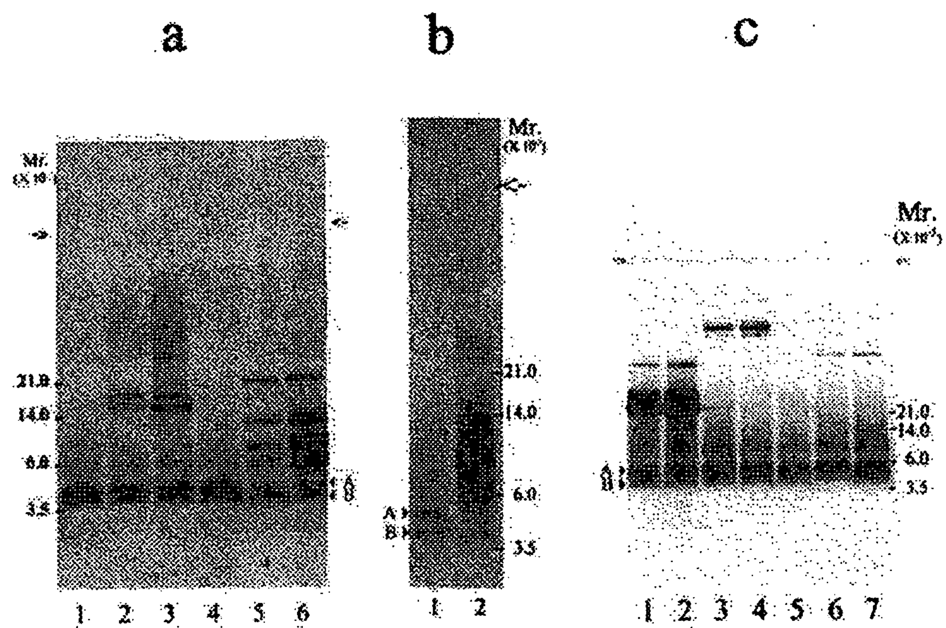


Figure 6. Sensitivity of Product R to Catabolic Enzymes

->, starting point of gel

Panel a, lane 1, Product R in Trypsin reaction buffer;
lane 2, Product R treated with Trypsin (0.05 mg/ml);
lane 3, Product R treated with Trypsin (0.025mg/ml;
lane 4, Product R in Chymotrypsin reaction buffer;
lane 5, Product R treated with Chymotrypsin (0.05mg/ml); and
lane 6, Product R treated with Chymotrypsin (0.025mg/ml).

Panel b, lane 1, Product R in Proteinase K reaction buffer; and
lane 2, Product R treated with Proteinase K (0.8mg/ml).

Panel c, lane 1, Product R treated with Ribonuclease A (0.5mg/ml);
lane 2, Product R treated with Ribonuclease A (1.0mg/ml);
lane 3, Product R treated with Alkaline Phosphatase (100units/ml);
lane 4, Product R treated with Alkaline Phosphatase (200units/ml);
lane 5, Untreated Product R;
lane 6, Product R treated with N-glycosidase F (20 units/ml); and
lane 7, Product R treated with N-glycosidase F (50 units/ml).

Note: All bands other than the 3.2 KDa and the 4.3 KDa bands represent either the enzymes themselves or the fragments of those enzymes.

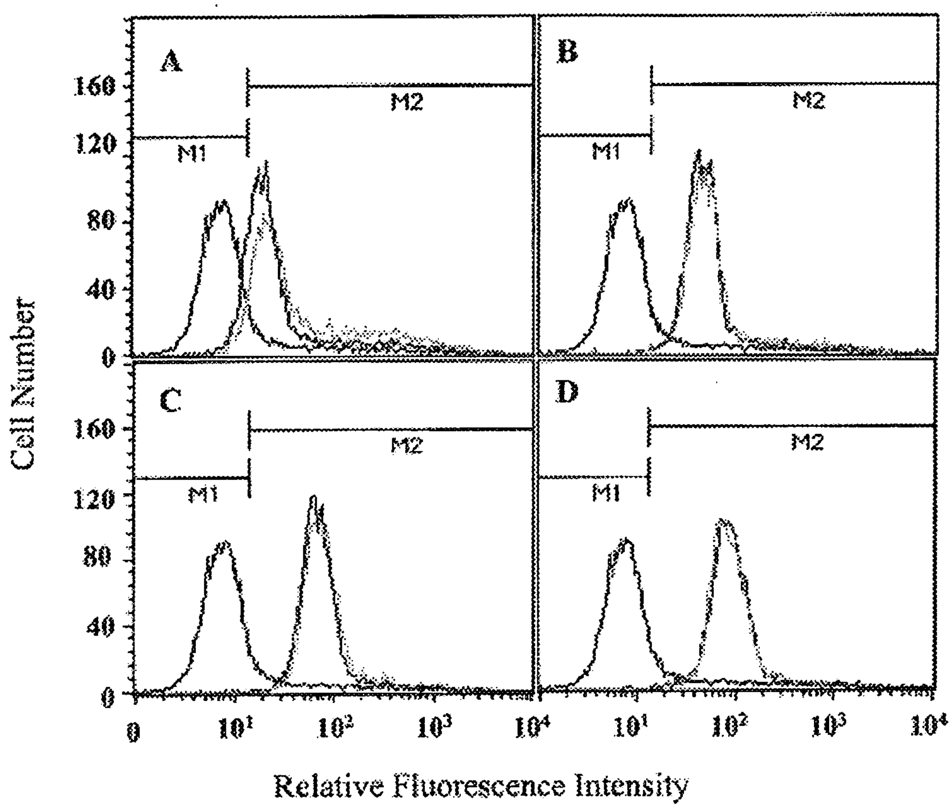


Figure 7. Effects of Product R upon phagocytosis of Dextran-FITC by U937 cells after 24 hr drug treatment. U937 cells were cultured with 5% Product R, or PBS as control, for 24 hr. Cells were harvested, washed and resuspended in binding buffer (RPMI 1640, 10% FCS, 1mM sodium pyruvate, 25 mM Hepes, and 1mg/ml glucose) containing 5 mg/ml Dextran-FITC. These cells were warmed to 37°C for A) 5 min, B) 15 min, C) 30 min , and D) 45 min to allow for phagocytosis. As a control cells were kept at 0°C for the entire incubation period. At the end of each incubation, cells were washed in binding buffer, fixed in 1% formaldehyde/PBS, and analyzed by flow cytometry using a FACSCalibur instrument (Becton Dickinson). The results were analyzed using CellQuest software supplied by the manufacturer. Graphs illustrated represent the relative fluorescence measured as a function of cell number. The following conditions are illustrated: green line = +PBS (control), purple line = +Product R, and black line = 0 min control.

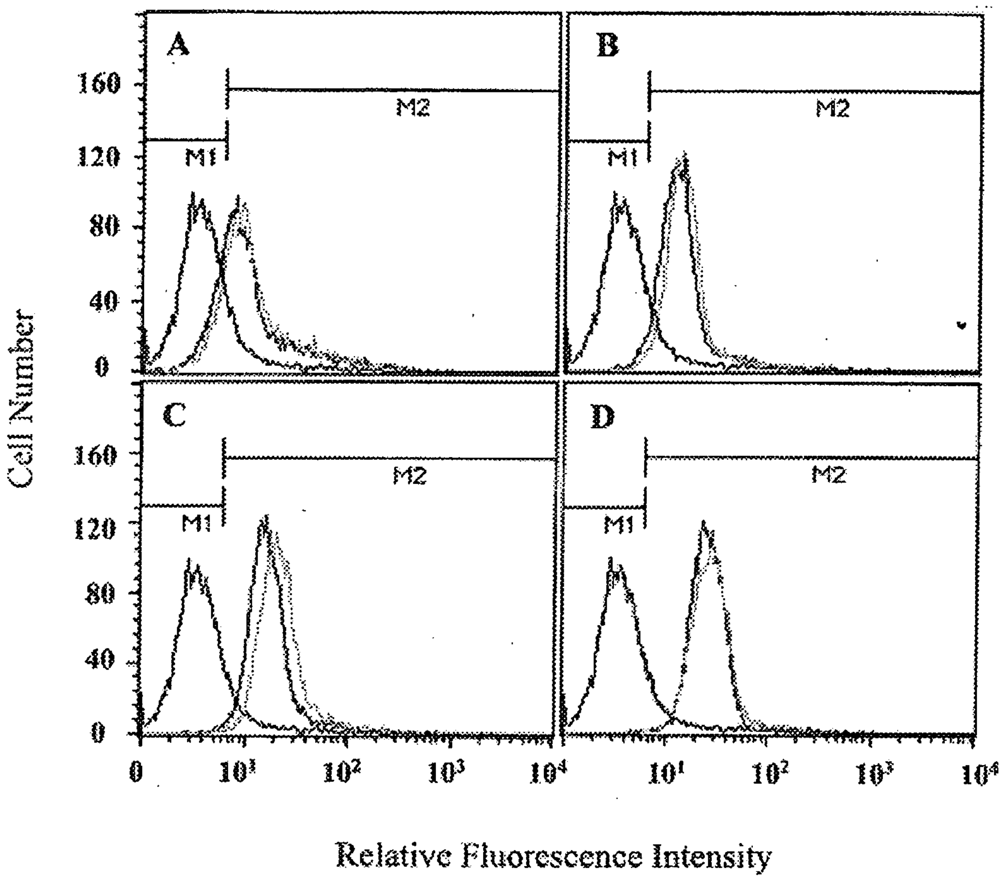


Figure 8. Effects of Product R upon phagocytosis of Dextran-Bodipy FL after an 8 day drug treatment. U937 cells were cultured with 5% Product R, or PBS, for 8 days. To measure phagocytosis, cells were treated similar to the description in Fig. 7, except cells were incubated with 5 mg/ml Dextran-BoDipy FL. These cells were warmed to 37°C for A) 5 min, B) 15 min, C) 25 min , and D) 40 min to allow for phagocytosis. Graphs illustrated represent the relative fluorescence measured as a function of cell number. The following conditions are illustrated: green line = +PBS (control), purple line = +Product R, and black line = 0 min control.

PREPARATION OF A THERAPEUTIC COMPOSITION

RELATED APPLICATIONS

This is a continuation-in-part application of the applicant's pending application Ser. No. 08/735,236, filed on Oct. 22, 1996, now abandoned. This application incorporates contents of the application Ser. No. 08/735,236 by reference in their entirety.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to an improved method of making a therapeutic composition, Product R[1], as hereinafter defined, which contains peptides and nucleotides. The components of Product R have molecular weights not more than 14 kilodaltons (KDa).

Product R was used as synonyms of RETICULOSE in some literature. For the purpose of the present application, Product R and RETICULOSE represent two distinct products.

2 . Description of the Related Art

The concept of an antiviral agent composed of peptones, peptides, proteins and nucleic acid was originated in 1934. After some years of experimentation, such an antiviral agent was modified by using bovine serum albumin in combination with peptone, and ribonucleic acid to produce an antiviral biotic agent which is nontoxic, free from anaphylactogenic properties and is miscible with tissue fluids and blood sera. The agent used to be described as a "lipopeptide-nucleic acid compound"[2] and registered under trademark RETICULOSE® by Chemico Laboratories, Inc. Physician Desk Reference, p 651, 1960. RETICULOSE® was reported as an antiviral agent for treating a variety of human viral infections, such as influenza, herpes, hepatitis A and B. It was then assumed that RETICULOSE® acts as an anti-viral agent at least by increasing leukogenesis, synthesis of antibodies and enhancing phagocytosis. RETICULOSE® was last sold in the United States in 1964.

The method of making RETICULOSE® had been kept as a trade secret by the manufacture until the issuance of U.S. Pat. No. 5,849,196, which discloses the method of making RETICULOSE®.

As disclosed in U.S. Pat. No. 5,849,196, the starting materials for making RETICULOSE® consist of, by weight, 40–50% of casein, 1–10% of blood albumin, 15–40% of beef peptone, 10–25% of RNA and 5–25% of sodium hydroxide. These starting materials are suspended in water which yields a ratio of proteins (casein, peptone and blood albumin) to water equals to about 4.3 to about 100 by weight. After an autoclaving treatment of the mixture of the starting materials, the resulting solution is filtered and pH is adjusted to approximately 8.5 and then to 7.8, after which the neutralized solution is filtered again. The pH is further adjusted to approximately 7.5 after the solution is diluted. Such process yields a mixture of peptides and nucleic acids having molecular weights in a range of approximately 1 to 25 KDa.

As taught by U.S. Pat. No. 5,849,196, the components over 15 KDa of the conventional composition of RETICULOSE® are more effective in treating viral diseases such as HIV, influenza virus, herpes simplex virus, etc. while the components in a range of approximately 1 to 15 KDa function as phagocytosis inhibitors.

However, the conventional methods suffers from several disadvantages: 1) the method does not ensure that each preparation produces the finished components having the same ratio, thereby the product is not reproducible; 2) the conventional method produces a wide range of the finished components, which makes the quality control of the preparation extremely difficult, if possible, because too many parameters need to be determined; 3) the presence of the higher molecular weight components, such as 25 KDa component, essentially peptides, increases the risk of hypersensitivity or immune reaction and renders the product less stable.

Therefore, it is desirable to have a product devoid of the deficiencies of conventional RETICULOSE® while maintaining its therapeutic properties.

SUMMARY OF THE INVENTION

Accordingly, an object of the present invention is directed to a novel therapeutic composition, Product R. Unlike RETICULOSE®, Product R is reproducible, highly stable and non-antigenic. Similar to RETICULOSE®, [redacted] Surprisingly, Product R has been proven to be effective in stimulating the production of chemokines including interferon-gamma, interleukin-6 and interleukin-1 (J. Investig Med 1996; 44:347–351), the production of red blood cells (U.S. Pat. No. 5,807,839), treating basal cell carcinoma (U.S. Pat. No. 5,902,786) and treating canine distemper viral infections (U.S. Pat. No. 5,807,840).

Another object of the present invention is directed to an improved method for making the novel therapeutic composition Product R. Product R according to the present improved method comprises novel components that generate a novel UV absorption spectrum and a novel molecular weight profile. Particularly, Product R comprises molecules having molecular weights not more than 14 KDa.

A further object of the present invention is to define the components of Product R based on chemical and physical methods.

Other objects and features of the present invention will become apparent from the following detailed description considered in conjunction with the accompanying drawings. It is to be understood, however, that the drawings are designed solely for purposes of illustration and not as a definition of the limits of the invention, for which reference should be made to the appended claims. It should be further understood that the drawings are not necessarily drawn to scale and that, unless otherwise indicated, they are merely intended to conceptually illustrate the structures and procedures described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawings will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

In the drawings:

FIG. 1 shows a representative ultraviolet absorption profile of Product R;

FIG. 2 shows a representative chromatogram of Product R obtained form a reverse phase HPLC analysis;

FIG. 3 shows a BioGel P-2 fractionation profile of Product R;

FIG. 4 shows the components of fraction I of the BioGel P-2 fractionation profile resolved on a 16% of SDS-Polyacrylamide gel electrophoresis (SDS-PAGE);

FIG. 5 shows the relative mass (Mr.) of the two major peptide components of Product R resolved on a 16% SDS-PAGE;

FIG. 6 is a 16% SDS-PAGE, showing the effects of a variety of catabolic enzymes on Product R;

FIG. 7 is a flow cytometric histograms, showing the effect of Product R on phagocytosis of Dextran-FITC; and

FIG. 8 is a flow cytometric histograms, showing the effect of Product R on phagocytosis of Dextran-BoDipyFL.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

Preparation of Product R

Generally, Product R is prepared according to the following manner.

First, the starting materials casein, beef peptone, RNA, BSA, and sodium hydroxide are suspended in proportions of, by weight, 35–50% (casein), 15–40% (beef peptone), 10–25% (RNA), 1–10% (BSA) and 5–25% (sodium hydroxide) in an appropriate volume of distilled water. All starting materials are generally available or otherwise can be readily prepared by a person of ordinary skill in the art. While any RNA is suitable for the intended purpose of the present invention, plant RNA is preferred and yeast RNA is the most preferred. The ratio of total proteins versus the volume of distilled water is generally about 1.5–2.5 to about 100 by weight, preferably about 2.2 to about 100 by weight. This means that every 1.5–2.5 grams of the total proteins are suspended in about 100 milliliters of distilled water.

All the starting materials are either generally commercially available or can be readily prepared by a person of ordinary skill in the art.

The suspension as prepared above is then autoclaved at a pressure of approximately 5–15 lbs., preferably 8–10 lbs. under an elevated temperature in a range, for example about 150°–300° F. preferably about 200°–230° F. over a period of approximately 2–10 hours, preferably more than 3 hours. As known to a person of ordinary skill in the art, under such conditions RNA may be completely hydrolyzed into nucleotides. After autoclaving, the solution is cooled down to room temperature, and then allowed to stay at a temperature of 3° to 8° C. for at least 12 hours to precipitate insoluble elements. Alternatively, the cooled solution may be centrifuged at a temperature below 8° C. to remove the precipitates.

The resulting solution is then filtered through a 2 micron and a 0.45 micron filters under an inert gas such as nitrogen or argon at a pressure of about 1–6 psi. In a similar manner the solution is filtered again through a pyrogen retention filter, preferably 0.2 micron.

After the above filtration, the solution may be cooled at 3 to 8° C. again for at least about 12 hours and filtered again in the same way as described above.

The resulting filtrate is then assayed for total nitrogen content using methods known to a person of ordinary skill in the art such as Kjeldahl method, J. G. C. D. Kjeldahl, Z. Anal. Chem., Vol. 22, p366 (1883), and its improvements. Based on the assay, the filtrate is then diluted with chilled distilled water to an appropriate volume having a preferred total nitrogen content ranging from 165 to 210 mg/ml.

The pH of the diluted solution is then adjusted with HCl to a physiologically acceptable pH, preferably to about 7.3 to 7.6, after which the diluted solution is filtered again through a 0.2 micron filter under an inert gas as described above.

Product R so produced contains essentially nucleotides, nucleosides and free nucleic acid bases of low molecular weights from a complete hydrolysis of RNA and small peptides from partial hydrolysis of the proteins. It is possible that the base hydrolysis of the proteins also produces free amino acids.

It is understood that the use of filtration technique is essentially to remove bacteria or other particles having similar size to or larger size than bacteria. Thus, any filter regardless its manufacturer or material from which it is made is suitable for the intended purpose. All filters used in the present process are widely available to a person of ordinary skill in the art.

The final filtrate is then filled and sealed into appropriate vials, such as 2 ml or 10 ml glass vials under an inert gas. The filled vials are autoclaved for final sterilization, after which they are ready for use.

In use, Product R is administered parenterally or topically to a patient in need as described in U.S. Pat. Nos. 5,807,839, 5,807,840 and 5,902,786, the contents of which are herein incorporated by reference in their entirety.

Characterization of Product R

The ultraviolet absorption spectrum:

FIG. 1 is a representative ultraviolet absorption spectrum of Product R measured in 1 cm path length quartz microcuvette (100 μl capacity) using a Shimadzu Model UV-1201 UV-VIS Spectrophotometer. Product R was diluted with distilled water by 100 fold. The spectrum is recorded between 220–320 nm and shows a maximum absorption at 260 nm and a trough at 235 nm. The ratio of the absorbance (A) at 260 nm over absorbance at 280 nm is 1.998 (±10%), and A at 260 nm over A at 230 nm is 1.359 (±10%).

The HPLC profile:

FIG. 2 is a representative chromatogram of Product R obtained from a reverse phase HPLC analysis using a Hewlett Packard 1100 HPLC system (Hewlett Packard Co.) that includes a binary pump (Model G1312A), a diode array detector (Model G1315A), a column thermostat (Model G 1316A), a thermostatted autosampler (Model G1329A), a sample thermostat and a vacuum degasser (Model G 1322A); and a stainless steel YMC-pack ODS-AQ S-5 uM column (YMC, Inc. 3223 Burnt Mill Dr., Wilmington, N.C. 28403) that has a size of 250×10 mm ID and pore size 120 A. The mobile phase consisting of a 0.1 M acetic acid: trietheylamine is prepared as follows: 6.0 ml of glacial acetic

acid are dissolved in 1000 ml of HPLC grade water. The stirred solution of acetic acid is titrated with triethylamine to pH 4.8. The solution is allowed to equilibrate overnight at room temperature and then filtered through a 0.45 μM pore size and 52 mm diameter filter. The pH of the solution is readjusted to pH 4.8 if necessary with the addition of triethylamine prior to use. The mobile phase is degassed by the vacuum degasser built into the HPLC flow system. 8 μl of Product R sample are injected, by means of an autosampler, into the column having a temperature set at 30° C. for each injection. The sample is then isocratically eluted from the column with 0.1 M acetic acid: triethylamine mobile phase (pH 4.8) at a rate of 1 ml per minute under a pump pressure of 92–102 bar. The chromatograms (UV absorbences at 260 nm) are run at 160 minutes per sample and the data are collected by the diode array detector and then analyzed using Hewlett-Packard HPLC ChemStation software. Graphical plots are generated and statistical analysis is conducted using the SigmaPlot program. The reverse phase HPLC under such conditions results in 13 characteristic HPLC peaks: A, B, C, D, E, F, G, H, I, J, K, L and M, each of which has a characteristic UV absorption profile (data not shown).

The BioGel P-2 gel filtration profile:

FIG. 3 shows a fractionation profile of Product R on a BioGel P-2 (Bio-Rad Laboratories Inc.) column having a size of 2.6 cm×55 cm packed size. After loading of Product R to the column, the column is eluted with a 0.1X PBS, preferably DULBECCO's PBS, free of calcium ion (Ca^{++}) and magnesium ion (Mg^{++}), at a flow rate of 0.5 milliliters per minute. 1X PBS contains 1.47 mM KH_2PO_4, 2.67 mM KCl, 138 mM NaCl and 8.1 mM Na_2HPO_4 $7H_2O$. The eluent passes through a "Uvcord SII" monitor, which is attached to a REC 101 chart recorder and fitted with a 254 nm filter, and is collected at 12 minutes per fraction in a "Frac 200" fraction collector. The gel filtration chromatography under such conditions results in 9 fractions: I, Ia, II, IIa, IIb, III, IIIa, IV and IVa. Each individual peak is compared with known nucleotides, nucleosides and free nucleic acid bases eluted at the same or very close to the volumes of respective fractions as shown in TABLE I. Known compounds having comparable values are shown in Remarks column.

TABLE I

	Experimental Value				
Peak	λmax	λmin	A_{260}/A_{280}	A_{260}/A_{230}	Remarks
Peak I	~275 nm	~255 nm	0.976	0.300	Mostly peptides and peptide conjugates
Peak Ia	~260 nm	~240 nm	1.636	0.943	Nucleoprotein and/peptide nucleic acid
Peak Is	~270 nm	~245 nm	1.258	0.939	Major component is CMP
Peak IIα	~260 nm	~230 nm	2.893	3.12	Major components are AMP, UMP
Peak IIβ	~250 nm	~225 nm	1.509	1.988	Major component is GMP
Peak IIa	~250 nm	~230 nm	1.257	1.176	Mixed components
Peak IIb	~270 nm	~250 nm	1.142	0.941	Major component is Cytidine
Peak III	~260 nm	~230 nm	2.695	3.664	Major component is Uridine
Peak IIIa	~260 nm	~225 nm	5.15	4.24	Major components are Uracil, Adenosine
Peak IV	~260 nm	~225 nm	5.406	3.892	Major component is Adenine
Peak IVa	~245 nm	~225 nm	1.016	1.285	Major component is Guanine

The fractions are then concentrated and analyzed by SDS-PAGE (see the following) on a 16% gel. Silver staining of the gel demonstrates that only fraction I shows essentially two major silverstainable bands having apparent molecular weights of 4.3 KDa, 5.2 KDa and a minor 7.6 KDa band as shown in FIG. 4.

The relative mass (Mr.):

FIG. 5 shows the relative mass (measurement of molecular weight) of the two major peptide components of Product R resolved on a 16% SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and stained by silver stain using 'SilverXpress'staining kit from NOVEX, following manufacturer-suggested protocol. Product R is resolved into two major silverstainable bands having apparent molecular weight of about 4.3 and about 5.2 KDa. A minor silverstainable component having molecular weight of about 7.6 KDa is also visible on an overloaded SDS-PAGE gel, and there may be trace amounts of other silverstainable peptides having molecular weights ranging from about 5 KDa to about 14 KDa. Coomassie Blue, a universal protein stain, stains the 4.3 KDa band extremely poorly. The three bands, 4.3 KDa, 5.2 KDa and 7.6 KDa, constitute more than about 90% of the peptides. Thus, Product R consists essentially of molecules having molecular weights below 8 KDa.

TABLE II shows the amino acid compositions of the 5.2 KDa and the 4.3 KDa components. Amino acid analysis of the 5.2 KDa band (sample A) and the 4.3 KDa band (sample B) was performed on a PE Bio-system 420 analyzer with automatic hydrolysis using standard pheno-iso-thiocyanite (PTIC) chemistry.

TABLE II

Amino acid	Sample A (~5.2 kDa) mol. %	Sample B (~4.3 kDa) mol. %
Aspartic acid	9.92	8.95
Glutamic acid	19.27	17.30
Serine	1.03	1.23

TABLE II-continued

Amino acid	Sample A (~5.2 kDa) mol. %	Sample B (~4.3 kDa) mol. %
Glycine	5.74	13.87
Histidine	2.58	3.11
Arginine	0.69	0.52
Threonine	0.73	1.78
Alanine	5.49	8.19
Proline	13.05	15.28
Tyrosine	4.39	3.37
Valine	9.95	5.39
Methionine	2.92	2.21
Isoleucine	5.47	3.45
Leucine	10.99	4.37
Phenylalanine	3.27	1.45
Lysine	5.12	9.53

The biochemical properties of the peptides:

Some biochemical properties of the silverstainable peptide components of Product R are analyzed using various catabolic enzymes, as described below:

The treatment with proteinase K (ICN Biochemicals):

Proteinase K is a non-specific broad spectrum protease that cleaves peptide bonds at the C-terminal of aliphatic, aromatic and hydrophobic amino acids. It may cleave all serum peptides completely at 50 μg/ml within one hour. A Product R sample is incubated in a reaction buffer having 10 mM Tris-HCl, pH 7.6; 0.5% of SDS; 1 mM $CaCl_2$; 100 μg/ml of proteinase K at 40° C. for 30 minutes and then subject to SDS-PAGE on a 16% gel as described above. Under such condition, the silver stain of Product R does not show significant change. However, when the amount of proteinase K is increased to 800 μg/ml and the incubation time is extended to one hour, the 5.2 KDa band disappears but there is no obvious change of the 4.3 KDa band.

The treatment with trypsin (Boehringer Mannheim, USA):

Trypsin is a serine protease, which specifically cleaves peptide bonds of lysine and arginine at the C-terminal at pH 7.5–9.0. A Product R sample is incubated in a reaction buffer having 100 mM Tris-HCl, pH 8.0 , 0.1% SDS and 250 μg/ml of sequencing grade trypsin at 25° C. for 19 and then subject to SDS-PAGE on a 16% gel. While serum proteins will be broken down to peptides smaller than 4.3 KDa under such reaction conditions, none of the silverstainable components of Product R are affected by trypsin.

The treatment with chymotrypsin (Boehringer Mannheim, USA):

Chymotrypsin is a serine protease that specifcally hydrolyses the peptide bonds of tyrosine, phenylalanine and tryptophan at C-terminals. It also cleaves peptide bonds of leucine, methionin, alanine, aspartic acid and glutamicacid at C-terminals at relatively lower rates. A Product R sample is incubated in a reaction buffer containing 100 mM Tris-HCl, pH 7.6, 10 mM $CaCl_2$ and 250 μg/ml of sequencing grade chymotrypsin at 25° C. for 19 hours and then subject to SDS-PAGE on a 16% gel. Chymotrypsin treatment significantly reduces the intensity of the 5.2 KDa and the 7.6 KDa bands but have no apparent effect on the 4.3 KDa band.

The treatment with pronase (Boehringer Mannheim, USA):

Pronase is a non-specific protease, acts on both native and denatured proteins. It breaks down virtually all proteins into their individual amino acids. The preparation contains various types of endo-peptidases such as srine and metalloproteases, exo-peptidases such as carboypepsidases, neutral protease and neutral and alkaline phosphatases. A Product R sample is incubated in a reaction buffer containing 100 mM Tris-HCl, pH 7.4; 10 mM $CaCl_2$; 0.1% SDS and 2 mg/ml of pronase from S. griseus at 40 ° C. for 75 minutes and then subject to SDS-PAGE on a 16% gel. All silver-stainable components disappear after such treatment of pronase.

The treatment with N-glycosidase F (Boehringer Mannheim, USA):

N-glycosidase F cleaves all types of asparagine bound N-glycans provided that the amino group and the carboxyl group are present in a peptide linkage and the oligosaccharide has the minimum length of the chitobiose core unit. A Product R sample is incubated in a reaction buffer containing 0.4X Dulbecco's PBS (where 1X PBS contains 1.47 mM KH_2PO_4, 2.67 mM KCl, 138 mM NaCl and 8.1 mM $Na_2PO_4 \cdot 7H_2O$), 0.1% SDS, 0.5% NP40 and 50 units/ml of recombinant N-glycosidase F at 37° C. for 4 hours and subject to SDS-PAGE on a 16% gel. The treatment N-glycosidase F does not alter the intensity of any of Product R bands on the 16% SDS gel. The resistance to N-glycosidase F indicates the lack of asparagine bound N-glycan, which is commonly observed in glycoproteins.

The treatment with ribonuclease A (ICN Biochemicals, USA)

Ribonuclease A is a pryimidine specific endoribonuclease that acts on single stranded RNA. A Product R sample is incubated in a reaction buffer containing 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, and I mg/ml of bovine pancreatic Ribonuclease A at 37° C. for about 1 hour and subject to SDS-Page on a 16% gel. Ribonuclease A does not alter the intensity of any of the Product R bands resolved by 16% SDS-PAGE gel. The resistance to ribonuclease A excludes the possibility of the presence of a RNA fragment attached to the peptide.

The treatment with alkaline phosphatase (Life Technologies, USA):

Calf thymus alkaline phosphatase (CIAP) is a phosphomonoesterase that hydrolyses 5'-phosphate groups from DNA, RNA and nucleotides. A Product R sample is incubated in a reaction buffer provided by the manufacturer of the enzyme and 200 units/ml CIAP at 37° C. for about one hour and subjected to SDS-PAGE on a 16% gel. CIAP does not alter the intensity of any of the Product R bands resolved by SDS-PAGE.

A summary of the above described treatments by catabolic enzymes is provided in the following TABLE III, and the results of the treatment are shown in FIG. 5, wherein "–" represents no substantial alteration of the stainable bands and "+" represents substantial alteration of the stainable bands.

TABLE III

Enzyme	Sensitivity of the Peptide Components of Product R (SDS-PAGE) 4.3 KDa	5.2 KDa	7.6 KDa
Proteinase K (100 μg/ml)	–	+/–	?*
(800 μg/ml)	–	+	?*
Trypsin (250 μg/ml)	–	–	–
Chymotrypsin (250 μg/ml)	–	+	+
Pronase (2 mg/ml)	+	+	+
N-glycosidase F (50 units/ml)	–	–	–
Ribonuclease A (1 mg/ml)	–	–	–
Alkaline Phosphatase (200 units/ml)	–	–	–

* This band is not clearly identified because of the presence of the enzyme fragments in that region.

The complexity of these enzymatic digestion patterns suggest that the peptide components of Product R may be conjugated with other molecules such a mono-nucleotides and/or carbohydrates, or intra/inter molecularly rosslinked.

RNA gel electrophoresis:

Neither agarose nor polyacrylamaide gel electrophoresis for nucleic acids generates any ethidium bromide stainable bands, indicating that there are no RNA fragments in Product R.

The effect of Product R on the phagocytosis:

FIGS. 7 and 8 are flow cytomeric histograms representing the cell-associated fluorescence, showing the effect of Product R on phagocytosis of Dextran-FITC or Dextran-BoDipyFL after 24 hours and 8 days of the Product R treatment, respectively. The effects of Product R on phagocytosis is tested using a human monocytic cell line, U937. The U937 cells are cultured in a medium having 5% of Product R, or 5% of PBS as a control, for 24 hours prior to the Dextran-FITC test, or 8 days prior to Dextran-BoBipyFl test. To measure phagocytosis, the cells are continuously fed with a phagocytic marker such as fluorescently-labeled Dextran-FITC for 5, 15, 30 and 45 minutes as indicated in FIG. 5, or Dextran-BoDipyFL for 5, 15, 25 and 40 minutes as indicated in FIG. 6 at 37° C. The quantity of a cell-associated fluorescence following phagocytic uptake is monitored using flow cytometry analysis according essentially to the method described by Sallusto, F. et al. (1995), J. Exp. Med., 182:389–400, which is herein incorporated by reference in its entirety. In these tests, the background values have been subtracted from those of the experimental samples and dead cells have been excluded from the data using propidium iodide exclusion.

Each of FIGS. 7 and 8 shows an overlay of the log fluorescence versus cell number for the PBS control (purple), the Product R treatment (green) and the background Dextran binding to cells (black). The purple curves (PBS control) are substantially overlapped with the green curves (Product R) at each time point, indicating that Product R does not inhibit phagocytosis of human monocytic cells.

Other biological functions of Product R:

Some of other known biological functions of Product R have been described in U.S. Pat. Nos. 5,807,840, 5,807,839 and 5,902,786; U.S. patent application Ser. Nos. 08/838,077, 08/838,069, 08/835,793, 08/835,794, 08/833,950, 08/837,992, 08/837,988, 08,838,070, 08/834,190, 08/835,791, 08/838,134, 08/839,651, 08/835,796, 08/964,250, 08/964,427, 08/923,516, 08/923,343, 08/922,888, [redacted] 09/007,565, 09/316,624, 09/316,374, 09/257,739 and the publication by Hirschman et al., J. Investig. Med. (1996; 44:347–351. These patents, patent applications and publication are herein incorporated by references in their entireties.

Conclusions

It is thus determined that the composition of Product R prepared according to the present described methods comprises nucleotides and peptides having molecular weights not more than 14 KDa, primarily not more than 8 KDa. The peptide components of Product R are unevenly distributed and typically located at two major silverstainable bands having molecular weights of 4.3 KDa, 5.2 KDa and a minor band of 7.6 KDa.

The UV absorption spectrum of Product R typically shows a maximum absorption at 260 nm and a trough at 235 nm, and the characteristic ratios of the absorbance of 260 nm over absorbance at 280 nm is 1.998 and at 260 nm over 230 nm is 1.359.

The HPLC profile of Product R comprises fractions of A, B, C, D, E, F, G, H, I, J, K, L and M as shown in FIG. 2.

The BioGel P-2 Gel filtration profile of Product R comprises fractions of I, Ia, II, IIa, IIb, III, IIIa, IV and IVa as shown in FIG. 3.

Comparison Between The Conventional Composition of Reticulose®And Product R

The composition of Product R as made according to the teachings of the present invention is compared with the conventional composition of RETICULOSE® with respect to their molecular weights (MW) and ultraviolet (UV) absorbancies (A) at wavelength of 230 nm, 260 nm and 280 nm, as shown in TABLE IV. While the components having molecular weights below 15 KDa of RETICULOSE® have been reported to inhibit the phagocytosis, the present application demonstrates that Product R does not inhibit the phagocytosis.

TABLE IV

		UV		
	MW	$A_{260/280}$	$A_{260/230}$	I/PH*
Product R	<14 KDa	1.998	1.359	No
RETICULOSE ®	1–25 KDa	2.839	1.198	Yes

*inhibition of phagocytosis by molecules having molecular weight below 15 KDa

Thus, Product R differs substantially from RETICULOSE® in their composition and bilogical functions.

TABLE V is a comparison between the relative amounts of the starting materials used for the preparations of the present therapeutic composition Product R and the conventional composition RETICULOSE®.

TABLE V

STARTING MATERIALS FOR INITIAL REACTION IN TEN LITERS	RETICULOSE ®	Product R
casein	250 grams	140 grams
beef peptone	150 grams	68.4 grams
serum albumin	15 grams	13 grams
RNA	80 grams	88 grams
NaOH	75 grams	66 grams

About 221 grams proteins are used in the initial reaction for the preparation of Product R, while about 415 grams for the preparation of RETICULOSE®. Thus, the initial protein concentration for the RETICULOSE® preparation is twice as much as that for the Product R preparation.

The following example only serves as an illustration of the process of making Product R and should not be construed as a limitation of the present invention.

EXAMPLE

Suspend about 35.0 g of casein, about 17.1 g of beef peptone, about 22.0 g of nucleic acid (yeast RNA), about 3.25 g bovine serum albumin in about 2.5 liters of water for injection USP at about 3 to 7° C. in a suitable container and gently stir until all the ingredients have been properly wet. Carefully add while stirring about 16.5 g of sodium hydroxide (reagent grade ACS) and continue stirring until sodium hydroxide completely dissolved. Autoclave at about 9 lbs. pressure and 200°–230° F. for a period of time until RNA is completely digested, for example, about 4 hours. At the end of the period, the autoclave is stopped and the reaction flask and contents are permitted to slowly cool to ambient temperature. Then cool for at least six hours at about 3°–8° C. The resulting solution is filtered through 2 micron and 0.45 micron filters using inert gas such as nitrogen or argon at low pressure (1–6 psi). In a similar manner the solution is filtered

again through 0.2 micron pyrogen retention filters. The resulting filtrate is sampled and assayed for total nitrogen. A calculation is then performed to determine the quantity of cooled water for injection to be added to the filtrate to yield a diluted filtrate with a nitrogen content between about 165–210 mg/100 ml, the final volume is approximately 5 liters. The pH is then adjusted with either concentrated HCl (reagent grade ACS) or 1.0 normal NaOH to about 7.3–7.6 range. The diluted solution is then filtered again through 0.2 micron filters with inert gas at low pressure. The final filtrate is then filled and sealed into 2 ml glass ampules while in an inert gas atmosphere. The amplules are collected and autoclaved for final sterilization at 240° F. and 14–16 pounds pressure for about 30 minutes. Following the sterilization cycle, the ampules with Product R are cooled and washed.

All quantities are subject to plus or minus 15% variation of pH, volume, and analytical adjustments.

Thus, while there have shown and described and pointed out fundamental novel features of the invention as applied to a preferred embodiment thereof, it will be understood that various omissions and substitutions and changes in the form and details of the devices illustrated, and in their operation, may be made by those skilled in the art without departing from the spirit of the invention. For example, it is expressly intended that all combinations of those elements and/or method steps which perform substantially the same function in substantially the same way to achieve the same results are within the scope of the invention. Moreover, it should be recognized that structures and/or elements and/or method steps shown and/or described in connection with any disclosed form or embodiment of the invention may be incorporated in any other disclosed or described or suggested form or embodiment as a general matter of design choice. It is the intention, therefore, to be limited only as indicated by the scope of the claims appended hereto.

We claim:

1. A peptide nucleic acid composition that absorbs light at wavelengths 230 nm, 260 nm and 280 nm so as to result in 260 nm/280 nm absorption ratio of about 1.998 and 260 nm/230 nm absorption ratio of about 1.359, comprising molecules of nucleotides resulting from a plant RNA and pleptides resulting from a mixture of casein, beef peptone and bovine serum albumin, said molecules having non-uniformly distributed molecular weights.

2. The composition of claim 1, wherein said nucleotides are mono-nucleotides.

3. The composition of claim 1, wherein said molecules have non-uniformly distributed molecular weights in a range from zero to substantially not more than 14 KDa.

4. The composition of claim 1, wherein said molecules have non-uniformly distributed molecular weights in a range from zero to substantially not more than 8 KDa.

5. The composition of claim 1, wherein said molecules have substantial concentrations at molecular weights of substantially 5.2 KDa and 4.3 KDa.

6. A method for preparing a peptide nucleic acid composition that absorbs light at wavelengths 230 nm, 260 nm and 280 nm so as to result in 260 nm/280 nm absorption ratio of about 1.998 and 260 nm/230 nm absorption ratio of about 1,359, said composition containing molecules of nucleotides and peptides having non-uniformly distributed molecular weights, comprising the steps of:

a. forming a mixture including a protein combination consisting oc casein, beef peptone and bovine serum albumin, a plant RNA and a base in water, wherein the ratio of said protein combination to said water is in a range from about 1.5/100 to about 2.5/100 by weight;

b. processing said mixture at an elevated temperature and an elevated pressure so as to form a solution and insoluble elements;

c. removing said insoluble elements;

d. diluting said solution with water; and

e. after performing steps b, c and d, adjusting the pH of said solution to a physiologically acceptable pH.

7. The method of claim 6, wherein the ratio of said protein combination of said water is about 2.2/100 by weight.

8. The method of claim 6, wherein said nucleotides are mono-nucleotides.

9. The method of claim 6, wherein said molecules have non-uniformly distributed molecular weights in a range from zero to substantially not more than 14 KDa.

10. The method of claim 6, wherein said molecules have non-uniformly distributed molecular weights in a range from zero to substantially not more than 8 KDa.

11. The method of claim 6, wherein said molecules have substantial concentrations at molecular weights of substantially 5.2 KDa and 4.3 KDa.

* * * * *